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(54) Title: AGENTS THAT DISRUPT PSD95 - nNOS INTERACTION, COMPOSITIONS CONTAINING THE SAME, AND THERAPEUTIC USES THEREOF

(57) Abstract: Agents capable of disrupting an interaction between neuronal nitrous oxide synthase (nNOS) and Post Synaptic Density Protein 95 (PSD95) are disclosed. The agents include small molecule compounds, natural product extracts, peptides, and fusion proteins. Compositions containing the disrupting agents, and use of the disrupting agents in the treatment of mammals suffering from conditions wherein nNOS-PSD95 interaction disruption provides a benefit, also are disclosed. Treatable conditions include pain, opiate tolerance, ischemic brain damage, neurological disorders, neurodegenerative disorders, Parkinson's disease, epilepsy, seizures, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and psychiatric disorders.

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AGENTS THAT DISRUPT PSD95-nNOS INTERACTION, COMPOSITIONS CONTAINING THE SAME, AND THERAPEUTIC USES THEREOF

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of provisional U.S. patent application No. 60/559,491, filed April 5, 2004.

FIELD OF THE INVENTION

The present invention relates to agents that disrupt interaction between neuronal nitric oxide synthase (nNOS) and the Post Synaptic Density Protein 95 (PSD95) and related proteins, and to compositions containing such a disrupting agent. The present invention also relates to methods of treating a disease or condition wherein disruption of PSD95-nNOS interaction provides a benefit. More particularly, the present invention relates to methods of treating acute and chronic pain, opiate tolerance, ischemic brain damage, neurological diseases, psychiatric disorders, and neurodegenerative diseases, for example, muscular dystrophy, Parkinson's disease, epilepsy, seizures, Huntington's disease, Alzheimer's disease, and amyotrophic lateral sclerosis, by administration of a therapeutically effective amount of a PSD95-nNOS disrupting agent to a mammal in need thereof.

BACKGROUND OF THE INVENTION

Numerous studies have shown that nitric oxide (NO) mediates diverse physiological functions associated with neurons. In the brain, NO acts as a neuromodulator to control behavioral activity, influence memory formation, and intensify responses to painful stimuli (J.E. Brenman et al., *Cur. Opin. in Neurobiol.*, *7*, 374-378 (1997); Z.D. Luo et al., *Curr. Rev. Pain*, *4*:459-466 (2000)).

NO biosynthesis in excitable tissues is regulated by increases in intracellular calcium, which activates NOS through enzyme dependence upon calmodulin. Although small amounts of NO synthesized during neural and skeletal muscle activity mediates physiological functions, an excessive production of NO can mediate tissue injury. For example, large amounts of NO produced during periods of cerebral ischemia mediate neuronal injury in various forms of stroke. Similar NO-mediated damage may account for neurodegeneration in other conditions, such as Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease (see reviews by Brenman and Luo, *supra*; also K.S. Christopherson et al., *J. Clin. Invest.*, *100*:2424-2429 (1997)). NO signaling also is perturbed in various muscle diseases, particularly in Duchenne muscular dystrophy.

Excessive amounts of NO are generated according to the following NMDA receptor-PSD95-nNOS pathway. The pathway also shows the effects of an increase in NO generation.

NMDA receptor activation → activation of PSD95-coupled nNOS → increased NO production → increases in cGMP (vasodilation), increases in neurotoxicity (neuronal damage), and increases in hypersensitivity of neurons (pain hyperalgesia).

This biological pathway occurs because N-methyl-D-aspartate (NMDA) receptors interact with the PSD95 protein, which in turn interacts with nNOS. NMDA receptor activation, therefore, is coupled to nNOS activation. PSD95 mediates the coupling of nNOS to NMDA receptors in the central nervous system (CNS).

The relationship between an NMDA receptor (NMDAR), PSD95, and nNOS is illustrated in Fig. 1. Fig. 1 shows that tails of the NMDA receptor interact with PSD95. PSD95 is a multidomain protein with three PDZ repeats, a Src homology (SH3) domain, and a 190-amino acid sequence having homology to yeast guanylate kinase (S.E. Cravin et al., *Cell*, *93*:495-498 (1998); K.S. Christopherson et al., *J. Biol. Chem.*, *274*:27467-27473 (1999); C.C. Garner et al., *Trends Cell Biol.*, *10*:274-280 (2000)). As further shown in Fig. 1, in the brain, the PDZ domain of nNOS targets nNOS to postsynaptic sites by binding to PDZ domains in PSD95. Importantly, NMDA receptors also occur at postsynaptic densities through binding to PSD95. PSD95 thereby functions as a molecular scaffold to physically link nNOS to NMDA receptors. The binding of PSD95 to nNOS couples NMDA receptor activity to the production of

nitric oxide (NO), a signaling molecule that mediates NMDA receptor-dependent excitotoxicity.

The arrangement of NMDA receptors, PSD95, and nNOS provides investigators various targets for reducing or eliminating an excessive production of NO, and thereby reducing the excitotoxicity of NMDA receptors. One target is the NMDA receptors, which mediate calcium ion (Ca^{2+}) influx into neurons. It is known that NMDA receptors contribute to the neuronal processes mediating prolonged nociceptive behaviors in pain models. Evidence suggests that the NMDA-type glutamate receptors play a pivotal role in the transmission of excitatory signals from primary sensory neurons to the brain through the spinal cord.

Inhibiting excitation, or activation, of the NMDA receptors can disrupt the NMDA receptor-PSD95-nNOS pathway. For example, neuropathic pain in man can occur following an injury to the peripheral or central nervous system arising from causes such as chemotherapy, traumatic injury, and herpes zoster infection. These neuropathies can be persistent, and are particularly problematic because they often are managed poorly by conventional opiate analgesics and nonsteroidal antiinflammatory drugs (NSAIDS).

Chronic pain can be maintained by a sensitization within the CNS that is mediated in part by the excitatory amino acids glutamate and aspartate binding to NMDA receptors. Therefore, NMDA receptor antagonists have been used in the treatment of neu-

ropathic pain (C.G. Parsons, *Eur. J. Pharm.*, *429*:71-78 (2001)). Effective NMDA receptor antagonists include various familiar drugs that also have NMDA antagonist properties, e.g., dextromethorphan, dextrorphan, memantine, ketamine, and amantadine.

These NMDA antagonists have been used to treat pain in patients, and particularly chronic and recurrent pain that has not responded to traditional therapies, such as opioids (C.N. Sang, *J. Pain Symptom Manage.*, *19*:S21-S25 (2000); D.J. Heweitt, *Clin. J. Pain*, *16*:S73-S79 (2000); M.E. Fundytus, *CNS Drugs*, *15*:29-58 (2001); C.G. Parsons, (2001), *supra*). The NMDA antagonists also have been used to reduce various types of neuropathic pain, including glossopharyngeal neuralgia, postherpetic neuralgia, central pain caused by spinal cord injury, stump pain, phantom limb pain, neuropathic cancer pain, limb pain after traumatic sciatic nerve injury, and surgery-induced nerve injury (see reviews: C.G. Parsons *et al.*, *Neuropharmacology*, *38*:735-767 (1999); C.G. Parsons, (2001), *supra*).

However, the clinical usefulness of some of these drugs is limited by a narrow therapeutic index attributed to unacceptable effects on mental functioning. In addition, these drugs also induce unacceptable side effects at analgesic doses, including hallucinations, dysphoria, memory impairment, psychotomimetic effects, ataxia, and disturbances of cognitive and motor function. Such adverse effects prohibit the widespread use of these NMDA receptor antagonists in the treatment of pain

(C.G. Parsons, (2001), *supra*). Therefore, in order to use an NMDA receptor antagonist in a treatment for neuropathic pain, new drugs having a reduced adverse side effect profile must be developed.

It also is known that neuropathic pain is relatively, and in some cases almost completely, resistant to opiate analgesics. This resistance is attributed to an interaction between the intracellular events that mediate central sensitization and events that regulate the sensitivity of the μ (mu) subtype of opiate receptor (J. Mao, *Brain Res. Rev.*, *30*:289-304 (1999)). Pain-evoked NMDA receptor activation increases cell responses to its stimuli, while it also decreases the efficacy of η (eta) receptor agonists. Thus, a patient with neuropathic hyperalgesia has a type of pain-evoked opiate tolerance, even in the absence of opiate treatment. Conversely, opiate tolerance appears to engage intracellular events similar to those engaged by pain-evoked NMDA receptor activation. Thus, the opiate-tolerant patient has a central sensitization-like hyperalgesia. Studies also show that NMDA receptor antagonists block sensitization to amphetamine and cocaine, as well as tolerance and dependence to ethanol and opiate analgesics in animal models (K. Elliott et al., *Neuropsychopharmacology*, *13*:347-356 (1995); Z. Wiesenfeld-Hallin, *Drugs*, *55*:1-4 (1998); D.D. Price et al., *J. Pain Symptom Manage.*, *19*:S7-S11 (2000)). NMDA receptor antagonists not only are able to prevent the development of morphine tolerance, but also reverse an established tolerance

even in the continuing presence of this opiate analgesic, and prevent the expression of withdrawal symptoms. Although some evidence exists that NMDA antagonists may synergize opiate analgesics, it also has been observed to be a mere additive, rather than synergistic, analgesic effect. The above-described adverse side effects associated with present-day NMDA receptor antagonists precluded their widespread clinical use in the treatment of chronic pain, and other conditions and diseases associated with the above-described NMDA receptor-PSD95-nNOS pathway.

However, specific inhibitors of NMDA receptor subtypes have proven beneficial in circumventing some side effects of broad spectrum NMDA receptor antagonists. Functional NMDA receptors are composed of heteromers containing NR1 subunit with one or more of the different NR2 subunits. (S.S. Nikam et al., *Curr. Pharm Des.*, *8*:845-855 (2002)). Of the four NR2 subunits, PSD95 predominantly binds to the NR2B receptor subunit (H.C. Kornau et al., *Science, 269*:1737-1740 (1995); Christopherson et al. (1999)). NR2B subunit has a more restricted localization than NR2A subunit, with higher concentration in forebrain and dorsal horn, areas that are important for nociception (S. Boyce et al., *Neuropharmacology, 38*:611-623 (1999)). Indeed, selective NR2B receptor antagonists have been shown to be efficacious in various animal pain models without motor dysfunction (S. Boyce et al. (1999); B.A. Chizh et al., *Trends Pharmacol. Sci.*, *22*:636-642

(2001); M. Zhuo, *Drug Discov. Today,* 7:259-267 (2002)). More recently, a NR2B selective receptor antagonist in a clinical trial was shown to significantly reduce pain intensity in subjects with spinal cord injury and monoradiculopathy (C.N. Sang et al., Program no. 814.9. 2003 Abstract viewer/itinerary planner. Washington D.C. Society for Neuroscience (2003)).

Another available target for disruption of the NMDA receptor-PSD95-nNOS pathway is the Post Synaptic Density Protein 95 (PSD95). It is known that NMDA receptor activity is unaffected by genetically disrupting PSD95 *in vivo* or by suppressing PSD95 expression *in vitro*. However, PSD95 deletion dissociates NMDA receptor activity from NO production and suppresses excitotoxicity.

PSD95 appeared to be a promising target for disrupting the NMDA receptor-PSD95-nNOS pathway either (a) by mutation, depletion, or elimination of PSD95, thereby eliminating the NMDR receptor-nNOS link, or (b) by disrupting the NMDR receptor-PSD95 interaction. This hypothesis was based on studies that showed suppression of PSD95 expression protected neurons against excitotoxicity produced by NMDA receptor activation, and PSD95 mRNA and protein are enriched in the spinal cord and selectively distributed in the superficial dorsal horn, where PSD95 overlapped with NMDA receptors. It also was found that PSD95 was required for NMDA receptor-mediated thermal hyperalgesia (K.F. Kitto et al., *Neurosci. Lett., 148*:1-5 (1992); Y.X. Tao et al.,

Neuroscience, *98*:201-6 (2000)) neuropathic pain, (F. Tao et al., *Neuroreport*, *12*:3251-5 (2001)), and mediated the role of NMDA receptors in determining the minimum alveolar anesthetic concentration of inhalational anesthetics (Y.X. Tao et al., *Anesthesiology*, *94*:1010-5 (2001)).

Thus, suppressing PSD95 expression uncoupled NO formation from NMDA receptor activation and also protected neurons against NMDA receptor toxicity, without affecting receptor function by mechanisms downstream from NMDA receptor activation and upstream from NO mediated toxic events. Overall, these studies showed that PSD95 imparts signaling and neurotoxic specificity to NMDA receptors through the coupling of receptor activity to nNOS.

Targeting the PSD95 protein represents an attractive therapeutic approach for diseases that involve NMDA receptor excitotoxicity because eliminating the PSD95 eliminates NMDA receptor coupling to nNOS, and as a result, excessive NO formation is precluded. In addition, eliminating PSD95 does not adversely affect other, beneficial functions of the NMDA receptors. This is an important feature because NMDA receptors mediate ischemic brain damage, for example, and totally blocking NMDA receptors is deleterious to mammals. In particular, investigators found that suppressing expression of PSD95 selectively attenuated excitotoxicity triggered via NMDA receptors, but not by other glutamate or Ca^{2+} channels. NMDA receptor function was unaffected because receptor expression, NMDA currents, and Ca^{2+}

loading were unchanged. Suppressing PSD95 blocked Ca^{2+}-activated NO production by NMDA receptors selectively, without affecting nNOS expression or function (R. Sattler et al., *Science, 284*:1845-8 (1999)).

Because mutation or suppression of PSD95 is therapeutically impractical, attention was directed to perturbing the PSD95 interaction with NMDA receptors to suppress excitotoxicity and ischemic brain damage (M. Aarts et al., *Science, 298*, 846-50 (2002)). It was found that peptides that disrupt the interaction of NMDA receptors with PSD95 dissociated NMDA receptors from downstream neurotoxic signaling without blocking synaptic activity or calcium influx. The peptides protected cultured neurons from excitotoxicity, reduced focal ischemic brain damage in rats, and improved their neurological function. This approach was expected to avoid the adverse side effects associated with blocking NMDA receptors. However, to date, no clinically effective NMDA receptor-PSD95 antagonist is known.

A third target for disrupting the NMDA receptor-PSD95-nNOS pathway is to inhibit NO formation by nNOS. NO acts as a neuromodulator in the CNS and participates in the regulation of diverse physiological processes including brain development, pain perception, neuronal plasticity, memory, and behavior. When produced in an excessive amount, however, NO transforms from a physiological neuromodulator to a neurotoxic effector. Overproduction of NO may occur from nNOS following persistent stimulation of

excitatory amino acid receptors mediating glutamate toxicity.

NO is a short-lived free radical, and regulation of signaling occurs largely at the level of NO biosynthesis (K.S. Christopherson et al., (1997), *supra*). Three mammalian NOS genes have been identified, and each forms NO from the guanidine nitrogen of L-arginine in a unique cytochrome P-450-type reaction that consumes reduced nicotinamide adenine dinucleotide phosphate (NADPH).

Because NO is membrane permeant, cells cannot sequester and regulate local NO concentration. Thus, unlike conventional transmitters that are stored in synaptic vesicles, the actions of which are mediated by binding to their receptors, and terminated by either reuptake mechanisms or enzymatic degradation, NO is produced on demand, directly reacts with an intracellular substrate, and terminates after the chemical reaction. Deregulation of nNOS in the brain is associated with overactivity of glutamate-type receptors, e.g., NMDA receptors, and contributes to neuronal damage in animal stroke models. nNOS, therefore, has been implicated in stroke and other neurodegenerative diseases in humans.

Thus, investigators sought a means to treat stroke and the other neurodegenerative diseases by providing (a) inhibitors of neuronal NO or (b) inhibitors of nNOS. The three isoforms of NOS, i.e., neuronal (nNOS, type I), endothelial (eNOS, type III), and inducible (iNOS, type II), are found

in the brain. Neuronal NOS is expressed in highly ramified neurons throughout the brain, including cerebellum, cerebral cortex, hippocampus, amygdale, and substantia nigra. Endothelial NOS is primarily localized in endothelial cells, although it has been detected in a small population of neurons. Inducible NOS is not found in healthy tissues, but can be expressed after brain insult in astrocytes, neurons, and endothelial cells.

The use of NOS inhibitors or mutant mice lacking all NOS isoforms provided evidence of the injurious effects of NO derived from the nNOS or iNOS isoforms in neurological diseases. In particular, a selective nNOS inhibitor, ARL17477, showed promising results in limiting ischemia-induced acute neuronal damage (P.E. Chabrier et al., *Cell Mol. Life Sci.*, *55*:1029-35 (1999)). In addition, inhibition of NO production by NOS inhibitors often results in suppression of hyperalgesia induced by tissue injury or chemical stimulations. However, it also has been found that neuron-derived NO plays a major role in regulation of blood flow. For example, in the brain, neuronal NO activity is associated with an increase in local blood flow. This response would be prevented by nNOS inhibitors. Therefore, total inhibition of nNOS would have a deleterious effect. In addition, to date, no clinically effective nNOS inhibitors are known. WO 03/029185 discloses compounds purported to be inhibitors of iNOS and/or nNOS.

SUMMARY OF THE INVENTION

Unlike the targets in the NMDA receptor-PSD95-nNOS pathway discussed above, the present invention is directed to agents that disrupt PSD95-nNOS interaction as a means to eliminate excessive NO production, and thereby treat a variety of diseases and conditions, particularly chronic pain.

More particularly, the present invention is directed to disrupting agents that dislocate nNOS from PSD95 and related proteins, and thereby interfere with the NMDA receptor-PSD95-nNOS pathway. The agents useful according to the invention are specific for disruption of PSD95-nNOS interaction and need not (and preferably do not) affect the catalytic activity of nNOS or the catalytic activity of other NOS isoforms. Disrupting agents of the present invention are expected to be excellent therapeutic agents in methods of treating pain, opiate tolerance, stroke, neurological diseases, neurodegenerative diseases, and other diseases and conditions wherein selective disruption of the NMDA receptor-PSD95-nNOS pathway provides a benefit.

The present invention, therefore, is directed to agents that disrupt interaction between PSD95 (and related proteins) and nNOS, and to compositions containing one or more of the agents. More particularly, the present invention is directed to methods of treating mammals, including humans, suffering from a disease or condition wherein disruption of PSD95-nNOS interaction provides a benefit.

Accordingly, one aspect of the present invention is to identify selective disrupting agents of PSD95-nNOS interaction, and thereby use the disrupting agents to reduce or eliminate the generation of excessive NO that adversely affects NMDA receptor-mediated pathways.

Another aspect of the present invention is to provide selective disrupting agents of PSD95-nNOS interaction selected from the group consisting of compounds of general structural formula (I), natural product extracts, peptides, and fusion proteins.

Another aspect of the present invention is to provide the use of a PSD95-nNOS disrupting agent and a pharmaceutically acceptable carrier for the manufacture of a medicament for the treatment of conditions and disorders wherein disruption of the NMDA receptor-PSD95-nNOS pathway provides a benefit.

Still another aspect of the present invention is to provide a method of treating a disease or a condition comprising administering a therapeutically effective amount of a PSD95-nNOS disrupting agent, or a composition containing the same, to a mammal in need thereof.

Yet another aspect of the present invention is to provide a method of treating nociceptive pain, neuropathic pain, opiate tolerance, ischemic brain damage, a neurological disease, a neurodegenerative disease, or a psychiatric disorder, by administering a therapeutically effective amount of PSD95-nNOS disrupting agent to a mammal including humans, in need thereof. Examples of specific

diseases and conditions that are treatable include, but are not limited to, Parkinson's disease, epilepsy, seizures, stroke, chronic pain, acute pain, Huntington's disease, amyotrophic lateral sclerosis, neuropathic pain, hyperalgesia, allodynia, traumatic brain injury, and muscular dystrophy, including Duchenne (or pseudohypertrophic) muscular dystrophy, Becker muscular dystrophy, limb-girdle muscular dystrophy, and fascioscapulohumoral muscular dystrophy.

Another aspect of the present invention is directed to administration of a PSD95-nNOS disrupting agent, alone or in combination with an opiate analgesic, to treat acute or chronic pain. More particularly, administration of an opiate analgesic in combination with a PSD95-nNOS disrupting agent potentiates the analgesic effect of the opiate analgesic, and, therefore, lowers the dose of opiate analgesic required to provide a desired pain-reducing effect. The reduced amount of opiate analgesic required to provide a desired pain-reducing effect also reduces the severity of various adverse side effects associated with opiate analgesic treatment. A PSD95-nNOS disrupting agent also can be used alone, either to treat chronic pain or to treat opiate tolerance attributed to excessive NO generated by the NMDA receptor-PSD95-nNOS pathway.

Still another aspect of the present invention is to provide a composition comprising an opiate analgesic, e.g., morphine, and a PSD95-nNOS disrupting agent for use in methods of treating pain.

The present invention also is directed to providing a method of reducing or reversing tolerance to an opiate analgesic in an individual undergoing an opiate analgesic therapy by administering a PSD95-nNOS disrupting agent to the individual. In the absence of an administered dose of a PSD95-nNOS disrupting agent, the opiate analgesic dose may have no effect, e.g., hyperalgesia, and/or would have to be increased over time to achieve the same pain-reducing effect. Administration of a PSD95-nNOS disrupting agent allows the opiate analgesic to be administered at a constant, or reduced, dose to achieve a desired pain treatment. The constant or reduced amount of opiate analgesic required to provide a desired pain-reducing effect thus reduces the severity of various adverse side effects associated with opiate analgesic treatment, and reduces the possibility of opiate analgesic dependence.

The present invention also provides a method for improved pain treatment. In particular, the present invention is directed to methods of using an opiate analgesic and a PSD95-nNOS disrupting agent to prevent and/or treat pain. More particularly, the present invention is directed to compositions containing morphine and a PSD95-nNOS disrupting agent, and to use of an opiate analgesic and a PSD95-nNOS disrupting agent, administered simultaneously or sequentially, in methods of treating pain and reducing or reversing opiate analgesic tolerance and dependence.

Another aspect of the present invention, therefore, is to provide a method and composition for ameliorating chronic or acute pain or a sensation thereof, while reducing the occurrence or severity of adverse side effects associated with opiate analgesic treatment.

Another aspect of the present invention is to reduce the problem of dependence and addiction associated with present opiate analgesics used to treat pain.

Still another aspect of the present invention is to provide a method of reducing or reversing opiate analgesic tolerance in an individual undergoing an opiate analgesic therapy by administering a therapeutically effective amount of a PSD95-nNOS disrupting agent to the individual.

Yet another aspect of the present invention is to provide an article of manufacture for human pharmaceutical use, comprising (a) a package insert, (b) a container, and either (c1) a packaged composition comprising an opiate analgesic and a PSD95-nNOS disrupting agent or (c2) a packaged composition comprising an opiate analgesic and a packaged composition comprising a PSD95-nNOS disrupting agent.

These and other aspects of the present invention will become apparent from the following detailed description of the preferred embodiments of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is an illustration summarizing the interactions between an NMDA receptor, PSD95, and nNOS;

Fig. 2 is a graph of % control vs. log of concentration of compound (1) to determine the *in vitro* IC_{50} value for compound (1);

Fig. 3 is a graph of % control vs. concentration (μM) of compound (1) showing the effect of compound (1) on NMDA vs. sodium nitroprusside (SNP)-induced cGMP elevation in cultured rat hippocampal neurons;

Fig. 4 is a graph of % neuroprotection vs. treatment showing the effect of compound (1) on ischemic-induced cell death in organotypic hippocampal slice cultures;

Fig. 5 is a graph of normalized V_{max} (mOD/-sec) vs. log of inhibition (M) showing the effect of compound (1) on rat brain nNOS;

Fig. 6 is a graph of % inhibition vs. concentration of compound (1) (fmoles/mouse) showing the effect of compound (1) on thermal hyperalgesia induced by NMDA in a mouse tail-flick model and an NMDA-induced scratching and biting behavior;

Fig. 7 is a graph of % maximum possible antihyperalgesic effect vs. compound dose (nmoles) for compound (1) and 7-nitroindazole (7-Ni) showing the effect of compound (1) on a model of chronic visceral hypersensitivity;

Figs. 8 and 9 contain plots of mechanical pressure threshold for paw withdrawal (grams) vs.

time (minutes) of the administration of compound (1) to illustrate the effect of vehicle (Fig. 8) and compound (1) (Fig. 9) on mechanical allodynia in neuropathic rats;

Fig. 10 is a graph of % control vs. concentration of Tat-nNOS(1-299) (μM) showing the effect of Tat-nNOS(1-299) on the PSD95-nNOS interaction *in vitro*;

Fig. 11 is a graph of % NMDA stimulation of cGMP level vs. log concentration of Tat-nNOS(1-299) (M) showing the effect of Tat-nNOS(1-299) on NMDA-induced cGMP;

Fig. 12 is a graph of % inhibition vs. concentration of Tat-nNOS(1-299) (fmoles/mouse) showing the effect of Tat-nNOS(1-299) on thermal hyperalgesia induced by NMDA in a mouse tail-flick model and on NMDA-induced scratching and biting behavior;

Fig. 13 contains plots of pressure (grams) vs. time (minutes) showing the effect of Tat-nNOS (7 nmol i.t.) on mechanical allodynia;

Fig. 14 contains plots of % inhibition vs. concentration of compound (1) administered intrathecally to mice showing tail flick latency and motor impairment five minutes after administration;

Fig. 15 contains plots of % inhibition vs. concentration of compound (1) administered intraperitoneally to mice, followed by NMDA administration, to determine tail flick latency and motor impairment; and

Fig. 16 contains plots of % inhibition vs. time (post injection) for administration of compound (1) intrathecally and intraperitoneally to mice, followed by intrathecal NMDA administration, for tail flick latency.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As described above, substantial research indicates that nNOS is critical in mediating neurotoxicity in stroke and various neurodegenerative diseases, as well as contributing to acute and chronic pain. Selective nNOS inhibitors have been difficult to identify because the cofactor and catalytic regions are highly conserved in the three NOS isoforms of NOS, i.e., the neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) forms. Of the three NOS isoforms, nNOS is the only isoform possessing a PDZ protein:protein interaction domain at its unique N-terminus. This N-terminus of nNOS binds to PSD95 and α1-syntrophin, which are scaffolding proteins expressed only in neurons and skeletal muscle, respectively. Disrupting neuron-specific PSD95-nNOS interaction provides an alternative approach to selectively inhibiting nNOS. Therefore, agents that disrupt PSD95-nNOS interaction can be excellent therapeutic agents for various diseases and conditions, such as stroke, neurological diseases, neurodegenerative diseases, pain, and opiate tolerance, for example.

Disrupting agents of interaction between PSD95 and nNOS illustrate the role of nNOS in the

treatment of pain, opiate tolerance, and other diseases and conditions mediated by excitotoxicity of NMDR receptors. The best nNOS inhibitors known to date exhibit only 10- to 30-fold inhibition selectivity for nNOS over the other two NOS forms. By using disrupting agents to dislocate nNOS from PSD95, and thereby interfere with the NMDA receptor-PSD95-nNOS pathway, the result is specific disruption of nNOS function, without affecting its catalytic activity, or the catalytic activity of other NOS isoforms. Such selective disrupting agents are excellent therapeutic agents in the treatment of neurological diseases, neurodegenerative diseases, neuropathic pain, and opiate tolerance, for example. The test results presented herein clearly demonstrate that disrupting agents of PSD95-nNOS interaction not only inhibit nNOS function in cells, but also in animal models.

As usual herein, the term "PSD95" refers to Post Synapatic Density Protein 95 and related proteins. Accordingly, the term encompasses PSD95 (also known as Synapse Associated Protein 90 kDa or SAP-90) and its related protein, such as PSD93/-chapsyn-110, SAP97/hdlg, and SAP-102. See S.N. Gomperts, *Cell*, *84*:659-662 (1996) and C.C. Garner et al., (2000)). Each of these proteins mediates the coupling of nNOS to NMDA receptors in the central nervous system (CNS).

The term "IC_{50} value" of a compound is defined as the concentration of the compound required

to produce 50% inhibition of biological or enzymatic activity.

The term "pharmaceutically acceptable carrier" is defined as to compounds suitable for use in contact with recipient animals, preferably mammals, and more preferably humans, and having a toxicity, irritation, or allergic response commensurate with a reasonable benefit/risk ratio, and effective for their intended use.

The term "alleviate" is defined as lessening, relieving, and/or mitigating pain. As such, pain experienced by an individual is made more bearable, but is not necessarily completely eliminated.

The terms "treating" and "treatment" is defined as preventing, lowering, stopping, or reversing the progression or severity of the condition or symptoms being treated. As such, the terms "treating" and "treatment" include both medical therapeutic and/or prophylactic administration, as appropriate.

The term "container" is defined as any receptacle and closure therefore suitable for storing, shipping, dispensing, and/or handling a pharmaceutical product.

The term "insert" is defined as information accompanying a product that provides a description of how to administer the product, along with the safety and efficacy data required to allow the physician, pharmacist, and patient to make an informed decision regarding use of the product. The package insert typically is prescribed by a regula-

tory agency as part of the approval or licensing of the medicine, and generally is regarded as the "label" for a pharmaceutical product.

The phrase "reducing or reversing opiate analgesic tolerance" is defined as the ability of a compound to reduce the dosage of an opiate analgesic administered to an individual to maintain a level of pain control previously achieved using a greater dosage of opiate analgesic.

The term "sensing" and "sensation" of pain are defined as an awareness of pain in an individual due to stimulation of a sense organ.

The term "nociceptive pain" is defined as pain caused by an injury to a body tissue. Examples of nociceptive pain include, but are not limited to, postoperative pain, cancer pain (e.g., pain from a tumor invading bones or organs, or pain resulting from cancer treatments, such as surgery or radiation therapy), or pain resulting from tissue damage (e.g., degenerative joint disease or fractures).

The term "neuropathic pain" is defined as pain caused by abnormalities in the nerves, spinal cord, or brain. In most types of neuropathic pain, indications of an original injury are gone and the reported pain is unrelated to an observable injury or condition. With neuropathic pain, certain nerves continue to send pain messages to the brain, even though there is no ongoing tissue damage. Neuropathic pain also may be caused by changes in neuronal connections or chemistry in the central nervous system. Neuropathic pain is different from nocicep-

tive pain caused by an underlying injury. Neuropathic pain is a type of chronic pain, but usually has distinct features from chronic pain of a musculoskeletal nature. Neuropathic pain may be felt as a burning or tingling sensation, or as a hypersensitivity to touch or cold. Neuropathic pain includes, but is not limited to, such syndromes as phantom limb pain, carpel tunnel syndrome, postherpetic neuralgia, reflex sympathic dystrophy, and causalgia.

The term "acute pain" is defined as a pain lasting less than about three to about six months, or a pain that is directly related to tissue damage, e.g., pain experienced from an incision or needle prick.

The term "chronic pain" is defined as a pain that lasts more than about three to about six months, or beyond the point of tissue healing. At least two different types of chronic pain problems exist, i.e., chronic pain due to an identifiable pain generator (e.g., an injury) and chronic pain with no identifiable pain generator (e.g., the injury has healed). Chronic pain usually is less directly related to identifiable tissue damage and structural problems. Nonlimiting examples of chronic pain include, but are not limited to, chronic back pain without a clearly determined cause, failed back surgery syndrome (i.e., continued pain after the surgery has completely healed), and fibromyalgia.

The term "inflammatory pain" is defined as a pain associated with the soft tissues, joints, and

bones of an individual. The cause of inflammatory pain can be, for example, osteoarthritis, rheumatoid arthritis, inflammatory myopathies, and muscle, tendon, or ligament injury due to sports or exercise.

The term "hyperalgesia" is defined as a shift of the pain stimulus-response function wherein an individual senses an excessive sensitivity to pain in relation to the intensity of the stimulus, i.e., an increased or exaggerated response to a normally painful stimulus. The term "hyperalgesia" is used for cases wherein an individual senses an increased pain response at a normal threshold, or at an increased threshold, e.g., in patients with neuropathy.

The term "allodynia" is defined as a condition wherein an ordinarily nonpainful stimulus evokes pain, i.e., a sensation of pain in response to a stimulus that normally does not provoke a pain sensation. Allodynia involves a change in the quality of a sensation, whether tactile, thermal, or of any other sort, wherein the original response to a stimulus was not a pain sensation, but the present response is a pain sensation. In contrast, hyperalgesia represents an augmented pain response to a normally painful stimulus.

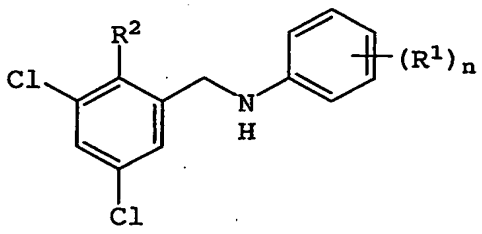
It also is understood that "a compound of formula (I)," or a physiologically acceptable salt or solvate thereof, can be administered as the neat compound, or as a pharmaceutical composition containing either entity.

A "therapeutically effective dose" refers to that amount of an active compound that, pursuant to a given mode of administration, results in achieving the desired effect. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The ratio between a toxic and a therapeutically affective does is termed the therapeutic index." Compounds which exhibit high therapeutic indices are preferred. Such data can be used in formulating a range of dosages for use in humans or other animals. The dosage of such compounds preferably lies within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed, and the route of administration utilized.

Disrupting agents, and pharmaceutical compositions containing the same, suitable for use in the present invention include those wherein the active ingredient is administered in an amount effective to achieve its intended purpose. More specifically, a "therapeutically effective amount" means an amount, pursuant to a given mode of administration, that is effective to prevent development of symptoms, or to alleviate the existing symptoms of, in the subject being treated. Determination of an effective amount of a given

active compound is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

One class of compounds found to be effective in the selective disruption of PSD95-nNOS interaction has a general structural formula (I):



(I)

wherein R^1, independently, is selected from the group consisting of C_{1-4}alkyl, halo, CF_3, OCF_3, $C(=O)R^a$, $C(=O)OR^a$, $N(R^a)_2$, $C(=O)N(R^a)_2$, $NR^aC(=O)N(R^a)_2$, OR^a, SR^a, NO_2, CN, $SO_2N(R^a)_2$, SOR^a, SO_2R^a, and OSO_2CF_3;

R^2 is hydro or OH;

R^a, independently, is selected from the group consisting of hydro, C_{1-4}alkyl, aryl, and heteroaryl; and

n is an integer 0 through 4,

wherein two R^1 groups can be taken together with the carbon atoms to which they are attached to form an optionally substituted 5- to 7-membered aliphatic or aromatic ring, and optionally containing one to three heteroatoms selected from the group consisting of oxygen, nitrogen, and sulfur,

and pharmaceutically effective salts, solvates, or prodrugs thereof.

As used herein, the term "alkyl" includes straight chained and branched hydrocarbon groups containing the indicated number of carbon atoms, typically methyl, ethyl, propyl, and butyl groups.

The term "halo" is defined herein to include fluoro, bromo, chloro, and iodo.

The term "aryl," alone or in combination, is defined herein as a monocyclic or polycyclic aromatic group, preferably a monocyclic or bicyclic aromatic group, e.g., phenyl or naphthyl. Unless otherwise indicated, an "aryl" group can be unsubstituted or substituted, for example, with one or more, and in particular one to four, halo, alkyl, trifluoromethyl, trifluoromethoxy, hydroxyalkyl, alkoxy, alkoxyalkyl, haloalkyl, nitro, amino, alkylamino, acylamino, alkylthio, and alkylsulfonyl. Exemplary aryl groups include phenyl, naphthyl, tetrahydronaphthyl, and the like, both unsubstituted and substituted. An abbreviation for phenyl, i.e., C_6H_5, is "Ph."

The term "heteroaryl" is defined herein as a monocyclic or bicyclic ring system containing one or two aromatic rings and containing at least one nitrogen, oxygen, or sulfur atom in an aromatic ring, and which can be unsubstituted or substituted, for example, with one or more, and in particular one to four, substituents, like halo, alkyl, trifluoromethyl, trifluoromethoxy, hydroxy, hydroxyalkyl, alkoxy, alkoxyalkyl, haloalkyl, nitro, amino, alkylamino, acylamino, alkylthio, and alkylsulfonyl. Examples of heteroaryl groups include thienyl,

furyl, pyridyl, oxazolyl, quinolyl, isoquinolyl, indolyl, triazolyl, isothiazolyl, isoxazolyl, imidizolyl, benzothiazolyl, pyrazinyl, pyrimidinyl, thiazolyl, and thiadiazolyl, both unsubstituted and substituted.

The term "hydro" is defined as -H.

The term "hydroxy" is defined as -OH.

The term "alkoxy" is defined as -OR, wherein R is C_{1-6}alkyl.

The term "amino" is defined as $-NH_2$, and the term "alkylamino" is defined as $-NR_2$, wherein at least one R is C_{1-6}alkyl and the second R is C_{1-6}alkyl or hydrogen.

The term "alkylthio" is defined as -SR, wherein R is C_{1-6}alkyl.

The term "alkylsulfonyl" is defined as RSO_2-, wherein R is alkyl.

The term "trifluoromethyl" is defined as $-CF_3$.

The term "trifluoromethoxy" is defined as $-OCF_3$.

The term "cyano" is defined as -CN.

The term "hydroxyalkyl" is defined as a hydroxy group appended to a C_{1-6}alkyl group.

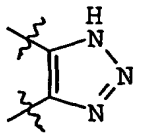
The term "alkoxyalkyl" is defined as a C_{1-6}alkyl group wherein a hydrogen atom has been replaced by an alkoxy group.

The term "haloalkyl" is defined as a C_{1-6}alkyl group substituted with one or more halo groups.

The term "nitro" is defined as $-NO_2$.

In a preferred embodiment, n is 0 to 3; R^1 is selected from the group consisting of halo, e.g., chloro and/or fluoro; OR^a, e.g., OH; $C(=O)OR^a$, e.g., $C(=O)OCH_3$ or $C(=O)OH$; C_{1-4}alkyl; NO_2; and $N(R^a)_2$, e.g., NH_2; and R^2 is OH.

In another preferred group of compounds of formula (I), two R^1 groups are taken together to form a 5- or 6-membered heteroaryl group, for example:



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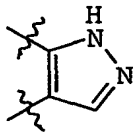

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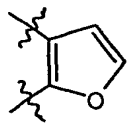

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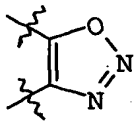

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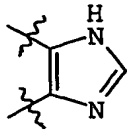

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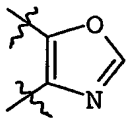

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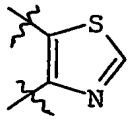
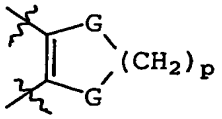
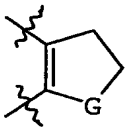


.

In another embodiment, two R^1 groups are taken together, with the phenyl ring to which they are attached, to form a bicyclic aromatic ring system, for example, naphthalene, indene, benzoxazole, benzothiazole, benzisoxazole, benzimidazole, quinoline, indole, benzothiophene, or benzofuran; or two R^1 groups are taken together to form

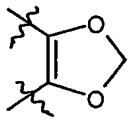


or



,

wherein p is 1 or 2, and G, independently, is $C(R^a)_2$, O, S, or NR^a. In a preferred group of compounds, p is 1 or 2, and G, independently, is $C(R^a)_2$ or O. Specific embodiments include, but are not limited to,



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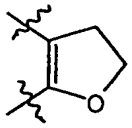
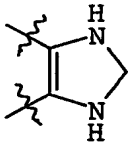


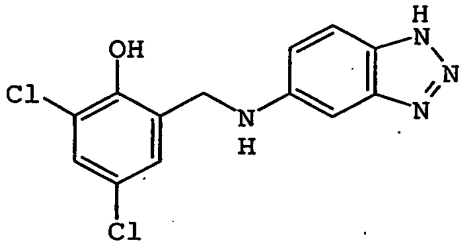
, and



.

When two R^1 groups are taken together to form a ring, the so-formed ring optionally can be substituted.

Specific examples of compounds of structural formula (I) include, but are not limited to,



(1)

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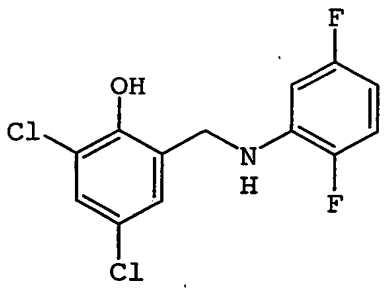
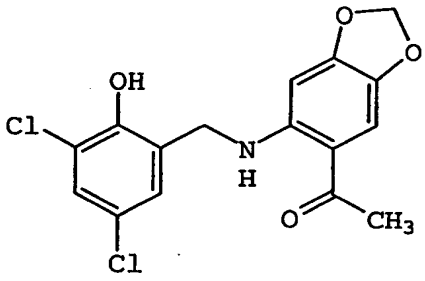
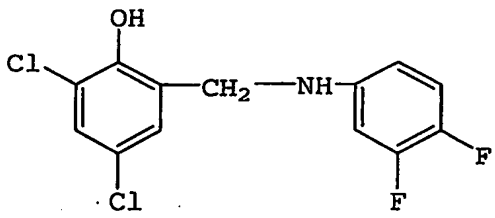


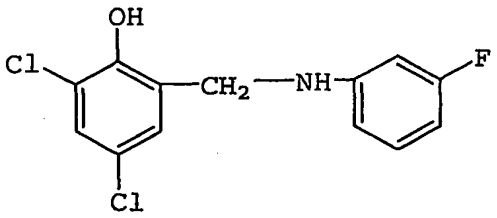
(2) , and

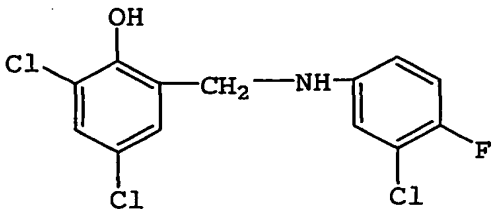


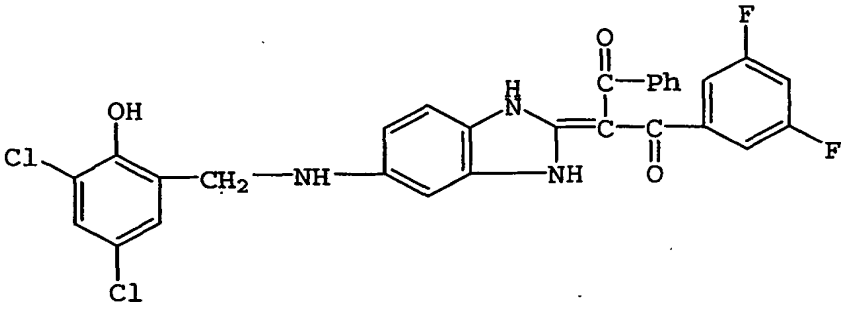
(3)

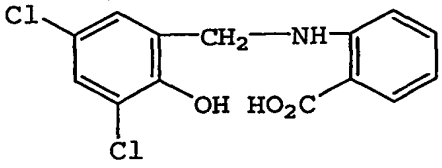
Additional compounds of structural formula (I) that can be used as an effective disruptor of the PSD95-nNOS interaction include, but are not limited to:

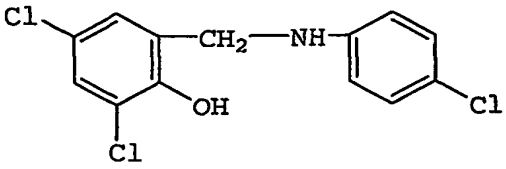












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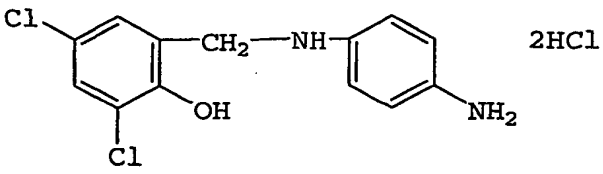
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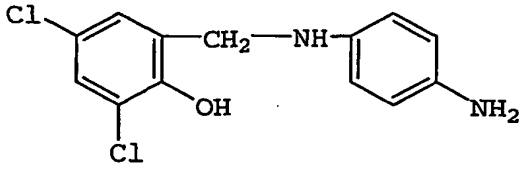
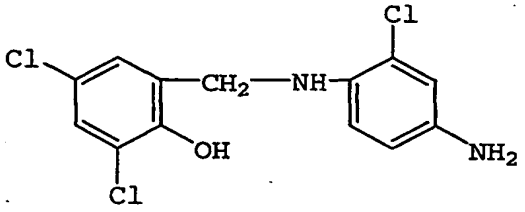
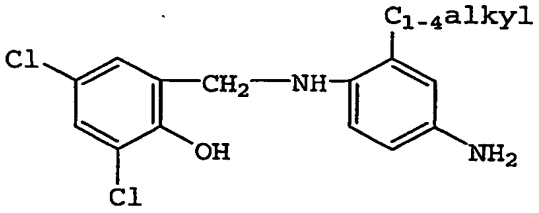
CH2
NH
2HCl
OH
NH2
Cl


Cl
CH2
NH
OH
NH2
Cl


Cl
Cl
CH2
NH
OH
NH2
Cl


C1-4alkyl
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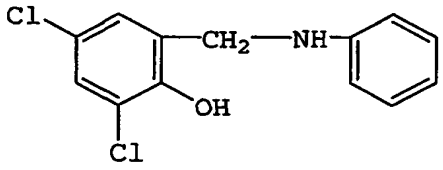
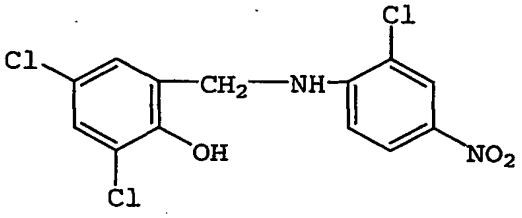
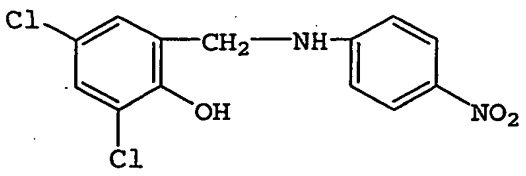
CH2
NH
OH
NH2
Cl


Cl
CH2
NH
OH
Cl

5


Cl
Cl
CH2
NH
OH
NO2
Cl



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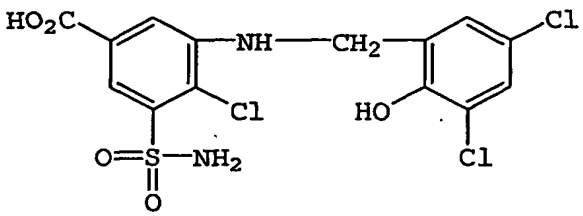

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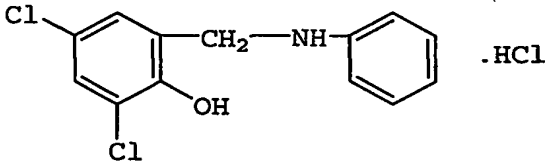


.HCl

Pharmaceutically acceptable salts of the compounds of formula (I) also can be used. These salts include acid addition salts formed with pharmaceutically acceptable acids. Examples of suitable salts include, but are not limited to, the hydrochloride, hydrobromide, sulfate, bisulfate, phosphate, hydrogen phosphate, acetate, benzoate, succinate, fumarate, maleate, lactate, citrate, tartrate, gluconate, methanesulfonate, benzenesulfonate, and p-toluenesulfonate salts. The compounds of formula (I) also can provide pharmaceutically acceptable metal salts, in particular alkali metal salts and alkaline earth metal salts, with bases. Examples include the sodium, potassium, lithium, magnesium, and calcium salts.

A compound of structural formula (I) also can be administered as a prodrug. The term "prodrug" as used herein refers to compounds that are rapidly transformed *in vivo* to a compound having structural formula (I), for example, by hydrolysis. Prodrug design is discussed generally in Hardma et al. (eds.), Goodman and Gilman's *The Pharmacological Basis of Therapeutics, 9th ed.*, pp. 11-16 (1996). A thorough discussion is provided in Higuchi et al., *Prodrugs as Novel Delivery Systems, Vol. 14*, ASCD Symposium Series, and in Roche (ed)., *Bioreversible Carriers in Drug Design*, American Pharmaceutical Association and Pergamon Press (1987). Briefly, administration of a drug is followed by elimination from the body or some biotransformation whereby biological activity of the drug is reduced or eliminated. Alternatively, a biotransformation process can lead to a metabolic by-product, which is itself more active or equally active as compared to the drug initially administered. Increased understanding of these biotransformation processes permits the design of so-called "prodrugs," which, following a biotransformation, become more physiologically active in their altered state. Prodrugs, therefore, encompass pharmacologically inactive compounds that are converted to biologically active metabolites. Prodrugs also can be active in the prodrug form.

To illustrate, prodrugs can be converted into a pharmacologically active form through hydrolysis of, for example, an ester or amide linkage, thereby introducing or exposing a functional group

on the resultant product. The prodrugs can be designed to react with an endogenous compound to form a water-soluble conjugate that further enhances the pharmacological properties of the compound, for example, increased circulatory half-life. Alternatively, prodrugs can be designed to undergo covalent modification on a functional group with, for example, glucuronic acid, sulfate, glutathione, amino acids, or acetate. The resulting conjugate can be inactivated and excreted in the urine, or rendered more potent than the parent compound. High molecular weight conjugates also can be excreted into the bile, subjected to enzymatic cleavage, and released back into the circulation, thereby effectively increasing the biological half-life of the originally administered compound.

PSD95-nNOS disrupting agents of general structural formula (I) generally have an IC_{50} value of less than about 200 μM, preferably less than about 100 μM, even more preferably less than about 50 μM, and usually from about 0.005 to 60 μM. Most preferably, a present PSD95-nNOS disrupting agent has an IC_{50} value of less than about 60 μM.

In addition to the compounds of general structural formula (I), various natural product extracts have been shown to successfully disrupt the PSD95-nNOS interaction. In particular, a screen of about 10,000 natural product extracts was performed. Soil samples containing both fungal and microbial sources were cultured in various broths. Ethanol extracts containing water soluble and lipid soluble

compounds were prepared, lyophilized, and reconstituted to original volume in 100% dimethyl sulfoxide (DMSO) prior to use. The extracts were screened at 10 µL/mL or at a 100-fold dilution from the original extracts (dissolved in 100% DMSO at 1 mL/mL of original extract volume). Seventy-five extracts were shown to have greater than 75% inhibition of the nNOS-PSD95 interaction. The IC_{50} values for these natural product extracts ranged from 0.2 to 20 µL/mL.

Thirty-three of these natural product extracts having an IC_{50} value less than 10 µL/mL were characterized further for specificity. Ten of the 33 extracts also inhibited the protein-protein interaction of AKAP79 and calcineurin, and thus were not investigated further. The remaining twenty-three extracts were specific for the PSD95-nNOS interaction and were refermented and fractionated to obtain an approximate estimate of molecule size. About one-third of these extracts were from *Actinomyces*, and the remainder came from fungal sources.

The extracts were applied to membrane filters (Millipore Ultrafree-MC centrifugal filters) having a weight average MW 5000 cut off. The filters then were centrifuged at 6000 rpm for 10 min. Activities of the remaining solution on the membrane (MW>5000) and of the filtered solution (MW<5000) were measured. It was found that all the inhibitory activities resided in molecular weight fraction of greater than 5000, suggesting that the active disrupting agents are high molecular weight compounds.

A natural product library containing molecules having a weight average molecular weight of less than 5000 also was tested. Nine extracts showed a consistent dose-dependent disruption of the nNOS-PSD95 interaction. After refermentation, activities still were found in the MW<5000 fraction for some of the extracts. The IC_{50} values of these extracts ranged from 24 to 94 µL/mL.

In addition to the compounds of structural formula (I) and natural product extracts that disrupt the protein-protein interaction between nNOS and PSD95, peptide inhibitors based on the C-terminal sequence of proteins that bind PSD95 also are useful. The PSD95 family of proteins is responsible for cell-surface clustering of Shaker-subfamily K^+ channels. This interaction is mediated by direct binding of the C-terminal cytoplasmic tails of the K^+ channel subunits to two PDZ domains in the PSD95 protein. Yeast dihybrid analysis and *in vitro* binding showed that the C-terminal 11 residues of K^+ channel, Kv1.4C, are sufficient to interact with PSD95, and that the last 5 amino acids (i.e., VETDV, SEQ ID NO: 1) are essential (E. Kim et al., *Nature, 378*:85-88 (1995); K.S. Christopherson, (1999), *supra*) reported that a peptide containing the last nine residues of the Kv1.4C channel can inhibit the protein-protein interaction between nNOS and PSD95.

Peptide inhibitors based on the last nine residues of Kv1.4C were generated and tested for their ability to inhibit nNOS-PSD95 interaction. Results are summarized in Table 1. This peptide,

when attached to the antennapedia sequence, has been reported to inhibit the protein-protein interaction of nNOS-PSD95 with similar potency to that for nNOS and syntrophin (an anchoring protein found in muscle). The critical region for the inhibitory activity of the nNOS-PSD95 interaction also was found to lie in the last five residues of Kv1.4C protein (VETDV). This short five amino acid (aa) peptide was found to be a potent inhibitor (IC_{50} value of about 2 μM), with a greater than 150-fold selectivity for the nNOS-PSD95 interaction over that for the nNOS-syntrophin interaction. The peptide inhibitors synthesized from this series all demonstrated a high selectivity for disruption of the nNOS-PSD95 interaction.

Structure-activity findings showed that the valine residue beginning the sequence VETDV may be N-acetylated, changed to proline, linked to the Tat protein, or deleted entirely, while retaining the ability of the pentapeptide to inhibit nNOS-PSD95 interaction. Inhibition is further maintained when the second acidic residue, i.e., glutamate, is replaced by the acidic homolog aspartate or the neutral amide analog glutamine, but not when replaced by the basic amino acids histidine or lysine. The threonine at the center of the sequence may be required for inhibition of the interaction of nNOS-PSD95 because conservative changes to serine or valine led to loss of activity. The exchange of threonine and its adjacent aspartate also eliminates activity. The acidic aspartate in the fourth posi-

tion can be altered to the neutral residue asparagine, the basic residue histidine, or the homologous acidic amino acid glutamate (if the second residue is changed from glutamate to aspartate), but not replaced with the hydrophobic amino acid leucine. The C-terminal valine residue can be replaced with other hydrophobic amino acids, such as leucine and isoleucine with maintenance of some activity. However, substitutions with glycine or alanine result in inactive peptides. Neutralization of the C-terminal carboxyl group by formation of the amide leads to no inhibition of nNOS-PSD95 interaction.

Because the N-terminal valine residue of VETDV can be deleted with only a ten-fold loss in potency, it is envisioned that a peptidomimetic drug design strategy starting with the simpler tetrapeptide ETDV (SEQ ID NO: 2) should provide inhibitors of the nNOS-PSD95 interaction. The ability to simultaneously neutralize two acidic residues with only a ten-fold loss in potency also is notable from a drug design standpoint. It further is anticipated that the stereochemistry of the amino acids at the backbone and in the side chains can affect potency and selectivity. In particular, it is theorized, but not relied upon herein, that the stereochemistry of the two asymmetric centers of the threonine may play a significant role in the activity of peptides containing this reside and in peptidomimetic molecules with a moiety based on threonine.

In general, the following penta- and tetrapeptides can be used to disrupt PSD95-nNOS interaction:

A-B-C-D-E,

wherein A is null, Pro, or Val, having a terminal NH_2 group that optionally can be acetylated or linked to Tat;

B is Glu, Gln, or Arg;

C is Thr;

D is Asp, Asn, or His;

E is Val, Leu, or Ile having a terminal $-CO_2H$ group; and wherein B can be Asp, if D is Glu.

Table 1
Summary of Peptide Disrupting Agents for the PSD95-nNOS Interaction

	nNOS-PSD95	nNOS-Syntrophin
Peptide	**IC_{50} (μM)**	**IC_{50} (μM)**
antennapedia-PSD95 BP RQIKIWFQNRRMKWK-KNAKAVETDV (SEQ ID NO: 3)	2.4	0.9
RQIKIWFQNRRMKWK-KAVEATA (SEQ ID NO: 4)	>100	
KNAKAVEDTA (SEQ ID NO: 5)	>100	
KAVEDTA (SEQ ID NO: 6)	>100	
NAKAVETDV (SEQ ID NO: 7)	50	>300
VETDV (SEQ ID NO: 1)	2 (n=6)	>300
VEDTV (mutant of above)	>300 (n=3)	>50
VEDTV (SEQ ID NO: 8)	>100	
VETDV-amide	>100	>100
acetyl-VETDV	2.68 (n=2)	
Tat-VETDV	0.24	>50
Tat-VEDTV mutant	>100	
Changing first position		
PETDV (SEQ ID NO: 9)	11	
Changing second position		
VQTDV (SEQ ID NO: 10)	2.07	>50
VDTDV (SEQ ID NO: 11)	30.49	>50
VRTDV (SEQ ID NO: 12)	about 50 (n=2)	>100
VKTDV (SEQ ID NO: 13)	>100 (n=2)	>100

Table 1
Summary of Peptide Disrupting Agents for the PSD95-nNOS Interaction

	nNOS-PSD95	nNOS-Syntrophin
Peptide	IC_{50} (μM)	IC_{50} (μM)
Changing third position		
VEVDV (SEQ ID NO: 14)	>100 (n=2)	>100
VESDV (SEQ ID NO: 15)	>100 (n=2)	>100
Changing fourth position		
VETNV (SEQ ID NO: 16)	4.897	>50
VQTNV (2nd and 4th) (SEQ ID NO: 17)	21.2 (n=2)	>100
VETLV (SEQ ID NO: 18)	>100 (n=2)	>100
VETEV (SEQ ID NO: 19)	>100 (n=2)	>100
VDTEV (2nd and 4th) (SEQ ID NO: 20)	27	
VETHV (SEQ ID NO: 21)	14	>100
Changing fifth position		
VETDL (SEQ ID NO: 22)	25 (n=2)	>100
VETDI (SEQ ID NO: 23)	14 (n=2)	>100
VETDG (SEQ ID NO: 24)	>100	
VETDA (SEQ ID NO: 25)	>100	
Four residues only		
ETDV (SEQ ID NO: 2)	18 (n=2)	>100

The Tat sequence also can be attached to any of these peptides to facilitate entry into cells. Tat peptides (based on the VETDV sequences shown in Table 1) that disrupt the nNOS-PSD95 interaction are envisioned to disrupt NMDA-increased cGMP production in primary neuronal cultures and to inhibit NMDA-nNOS dependent hyperalgesia in animal pain models.

Another approach to disrupting PSD95-nNOS protein-protein interaction is to administer a catalytically inactive nNOS that retains the PSD95 binding region. The first 300 residues of nNOS (1-299) contain the three PDZ binding regions critical for interacting with the PDZ domains of PSD95. How-

ever, this nNOS (1-299) does not contain the catalytic region of NOS and is not active. The Tat sequence (S.R. Schwarze et al., *Science, 285*:1569-1572 (1999)) of HIV protein therefore is fused with nNOS (1-299) to generate Tat-nNOS (1-299). This fusion protein is envisioned to enter cells and displace the wild type catalytically active nNOS from binding to PSD95. This displacement disrupts the nNOS targeting to PSD95, uncouples nNOS from NMDA receptor, and thus inhibits the NMDA receptor-PSD95-nNOS pathway.

To generate Tat-nNOS (1-299), nNOS (1-299) is inserted into pRSET vector with the Tat sequence already engineered to generate an N-terminal Tat sequence to the resulting fusion protein. This fusion protein inhibited *in vitro* binding of nNOS to PSD95 with an IC_{50} value of 1 μM. It also inhibited NMDA-increased cGMP production in primary cultures of hippocampal neurons with an EC_{50} value of 3 μM. When Tat-nNOS was administered intrathecally (i.t.) into mice, it inhibited NMDA-induced hyperalgesia with EC_{50} value of pmole/mouse.

The core nNOS region for binding PSD95 resides in amino acids 16-130 (Christopherson et al., (1999)). Deletion of residues N- or C-terminal to this region results in loss of binding. It is envisioned that Tat-nNOS (16-130) will inhibit NMDA-nNOS-dependent pathways in cell cultures and in animal models, while Tat-nNOS sequences that do not inhibit the nNOS-PSD95 interaction will have no effect. The same applies for mutations on nNOS that

would disrupt nNOS-PSD95 interactions. Mutations of residues E108, T109, T110, or F111, i.e., residues on nNOS that are critical for PSD95 binding (H. Tochio et al., *J Mol Biol, 303*:359-370 (2000)), are not expected to displace the existing nNOS-PSD95 interaction, because these mutant proteins do not bind to PSD95. These mutant proteins thus are not envisioned to inhibit NMDA-nNOS-dependent pathways in cell cultures or in animal studies.

The agents of the present invention are potent and selective disruptors of PSD95-nNOS interaction. Thus, the PSD95-nNOS disrupting agents are of interest for use in therapy, specifically for the treatment of a variety of conditions where selective inhibition of the NMDA-PSD95-nNOS pathway is considered to be beneficial.

Disruption of PSD95-nNOS interaction is a particularly attractive target to attenuate excitotoxicity of NMDA receptors. A potent and selective disruption of PSD95-nNOS interaction reduces or eliminates the production of excessive amounts of NO in the NMDA receptor-PSD95-nNOS pathway, which is beneficial in the treatment of various disease states.

An especially important use is the treatment of acute and chronic pain. Excessive production of NO in the NMDA receptor-PSD95-nNOS pathway increases neurotoxicity and hypersensitivity of neurons, which leads to hyperalgesia. Opiate analgesics are marginally effective, or ineffective, in the treatment of such pain states, thus the condi-

tion has a natural tolerance to opiate analgesics. Furthermore, if an opiate analgesic has an effect in certain individuals, the dose of analgesic typically is relatively high, which in time leads to tolerance and possible dependence on the opiate analgesic.

It is envisioned, therefore, that PSD95-nNOS disrupting agents of the present invention are useful in the treatment of acute and chronic pain conditions, including neuropathic pain. A disrupting agent of the present invention can be used, either alone to reduce hyperalgesia and opiate tolerance, or in combination with an opiate analgesic such that the amount of the opiate analgesic dose can be reduced to achieve a predetermined reduction in pain.

With respect to the treatment of pain, pain indications have been grouped in two ways, i.e., (a) acute, chronic, and neuropathic pain, wherein neuropathic pain is considered either a subset of chronic pain or distinct from it), and (b) grouping pain indications by where the pain is felt.

Regardless of the pain indication grouping that is used, the present invention is useful in the treatment of pain originating from soft tissues, joints, and bones, for example, acute and postoperative pain; osteoarthritis; rheumatoid arthritis; and pain of muscles, tendons, and ligaments (including, but not limited to, trauma and sports/exercise injuries, inflammatory myopathies, muscle cramps, myalgia of neurogenic origin, drug-induced myalgia, myalgic encephalomyelitis and muscle pain of uncer-

tain cause); chronic back pain; upper extremity pain; and fibromyalgia.

The present invention also is useful in the treatment of deep and visceral pain including, but not limited to, abdominal pain (including chronic abdominal pain, for example), pain from acute appendicitis, mesenteric lymphadenitis, Crohn's disease, tubo-ovarian disorders, renal disorders, acute and chronic pancreatitis, peritonitis, AIDS, intestinal obstruction, opiate withdrawal, atypical gastro-oesophageal reflux disorders, gastric ulcer, irritable bowel syndrome, constipation); heart, vascular, and haemopathic pain; chronic pelvic pain; obstetric pain; and genitourinary pain.

The invention also can treat pain in the head area, for example, orofacial pain (including dental pain, periodontal pain, gingival pain, and mucosal pain); trigeminal, eye, and ear pain; and headache (including migraine and headache associated with head trauma, vascular disorders, substance withdrawal, or a metabolic disorder).

Nerve and root damage pain also can be treated by the present invention. For example, phantom pain and other phenomena after amputation; peripheral neuropathies, complex regional pain syndrome; and nerve root disorders (e.g., nerve root compression and root damage); and arachnoiditis are treatable by the present invention. Additional pain states treatable by the present invention include central nervous system pain, e.g., central pain or

spinal cord injury, and cancer pain, attributed either to the cancer or a treatment for the cancer.

Specific pain types and pain syndromes treatable by the present invention include, but are not limited to,

chronic and acute arthritis pain;
chronic and acute back pain;
cancer pain;
failed back syndrome;
fibromyalgia;
herpes zoster (shingles);
intercostal pain;
myofascial pain dysfunction syndrome;
neck, arm, and shoulder pain;
neuralgias/neuropathy;
occipital neuralgia;
phantom limb pain;
postherpetic neuralgia;
postoperative pain;
reflex sympathetic dystrophy;
thoracic pain;
sacroiliac "SI" joint pain;
headaches (including migraine);
posttrauma pain;
sciatica;
facial pain (trigeminal neuralgia);
musculoskeletal pain;
complex regional (reflex sympathetic dystrophy or causalgia);
degenerative joint disease;
spinal stenosis;

fibrositis;
trigger points;
diabetic neuropathy;
disc disease;
amputation pain;
bladder pain;
dysuria;
radiculopathies;
arachnoiditis;
labor (of childbirth) pain;
pelvic pain; and
oral pain.

Although the PSD95-nNOS disrupting agents of the present invention are envisioned primarily for the treatment of pain in mammals, they also can be used for the treatment of other disease states. A further aspect of the present invention, therefore, is to provide a PSD95-nNOS disrupting agent for use in the treatment of neurological disorders and neurodegenerative disorders, or other disorders mediated by the NMDA receptor-PSD95-nNOS pathway. Conditions and diseases treatable by the present invention include, but are not limited to, ischemic brain damage, stroke, Parkinson's disease, Huntington's disease, seizures, epilepsy, amyotrophic lateral sclerosis, psychiatric disorders, Duchenne (or pseudohypertrophic) muscular dystrophy, traumatic brain injury, Becker muscular dystrophy, limb-girdle muscular dystrophy, and fascioscapulohumeral muscular dystrophy. In the treatment of these conditions and diseases, a PSD95-nNOS disrupting agent can be

used alone, or in combination with a second therapeutic agent useful in the treatment of the disease or condition. Therapeutic agents useful in the treatment of a particular disease or condition are well known to persons skilled in the art.

In one embodiment, the present invention is directed to the simultaneous or sequential administration of an opiate analgesic and a PSD95-nNOS disrupting agent to prevent and/or treat pain. In particular, the administration of morphine and a PSD95-nNOS disrupting agent potentiates the analgesic effect of morphine, and, therefore, the dose of morphine can be reduced, while providing an analgesic effect equivalent to administering a higher dose of morphine alone. The reduced dose of morphine also reduces adverse side effects associated with morphine administration, and can significantly reduce the addiction potential of morphine in susceptible individuals.

The present invention also is directed to the administration of a PSD95-nNOS disrupting agent to an individual to reduce or reverse opiate analgesic tolerance in the individual. By reducing or reversing tolerance to an opiate analgesic, an opiate analgesic can be used in the treatment of hyperalgesia. In addition, the administration of a PSD95-nNOS disrupting agent allows the dose of opiate analgesic to remain constant, or to be reduced, while maintaining the desired pain-reducing effect. The occurrence of adverse side effects attributed to the opiate analgesic can be reduced, and the possi-

bility of opiate analgesic dependence is reduced. The analgesic and disrupting agent can be administered simultaneously or sequentially to achieve the desired effect of pain treatment or reduction or reversal of opiate analgesic tolerance.

An opiate analgesic utilized in the present invention can be one or more opium alkaloid or semisynthetic opiate analgesic. Specific opiate analgesics include, but are not limited to, (a) opium; (b) opium alkaloids, such as morphine, morphine sulfate, codeine, codeine phosphate, codeine sulfate, diacetylmorphine, morphine hydrochloride, morphine tartrate, and diacetylmorphine hydrochloride; and (c) semisynthetic opiate analgesics, such as dextromethorphan hydrobromide, hydrocodone bitartrate, hydromorphone, hydromorphone hydrochloride, levorphanol tartrate, oxymorphone hydrochloride, and oxycodone hydrochloride. Other opioids include, but are not limited to, fentanyl, meperidine, methodone, alfentanil, remifentanil, sulfentanil, and propoxyphene.

In accordance with another important feature of the present invention, an opiate analgesic is present in a composition, or is administered, with a PSD95-nNOS disrupting agent in a weight ratio of analgesic-to-antagonist of about 0.01:1 to about 100:1, preferably about 0.02:1 to about 50:1, and most preferably about 0.1:1 to about 10:1. This ratio depends upon the type and identity of opioid analgesic and disrupting agent being used. The ratio of analgesic-to-disrupting agent that is ad-

ministered is dependent upon the particular analgesic and disrupting agent, and the origin and severity of the pain being treated. This ratio can be readily determined by a person skilled in the art to achieve the desired reduction in pain.

The opiate analgesic and disrupting agent can be administered to mammals in methods of treating pain. The opiate analgesic and disrupting agent can be formulated in suitable excipients for oral administration, or for parenteral administration. Such excipients are well known in the art. The active agents typically are present in such a composition in an amount of about 0.1% to about 75% by weight, either alone or in combination.

Pharmaceutical compositions containing the active agents, i.e., opiate analgesic and PSD95-nNOS disrupting agent, of the present invention are suitable for administration to humans or other mammals. Typically, the pharmaceutical compositions are sterile, and contain no toxic, carcinogenic, or mutagenic compounds that would cause an adverse reaction when administered.

The method of the invention can be accomplished using the active agents as described above, or as a physiologically acceptable salt, prodrug, or solvate thereof. The active agents, salts, prodrugs, or solvates can be administered as the neat compounds, or as a pharmaceutical composition containing either or both entities.

The active agents can be administered by any suitable route, for example by oral, buccal,

inhalation, sublingual, rectal, vaginal, intracisternal through lumbar puncture, transurethral, nasal, percutaneous, i.e., transdermal, or parenteral (including intravenous, intramuscular, subcutaneous, and intracoronary) administration. Parenteral administration can be accomplished using a needle and syringe, or using a high pressure technique, like POWDERJECT™. Administration of the active agents can be performed before, during, or after the onset of pain.

The exact formulation, route of administration, and dosage is determined by an individual physician in view of the patient's condition. Dosage amounts and intervals can be adjusted individually to provide levels of active agents that are sufficient to maintain therapeutic or prophylactic effects.

The amount of active agents administered is related to the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration, and the judgment of the prescribing physician.

Specifically, for administration to a human in the curative or prophylactic treatment of pain, oral dosages of an opiate analgesic and PSD95-nNOS disrupting agent, individually generally are about 10 to about 200 mg daily for an average adult patient (70 kg), typically divided into two to three doses per day. Thus, for a typical adult patient, individual tablets or capsules contain about 0.1 to about 200 mg opioid analgesic and about 0.1 to about

50 mg disrupting agent, in a suitable pharmaceutically acceptable carrier, for administration in single or multiple doses, once or several times per day. Dosages for intravenous, buccal, or sublingual administration typically are about 0.1 to about 10 mg/kg per single dose as required. In practice, the physician determines the actual dosing regimen that is most suitable for an individual patient, and the dosage varies with the age, weight, and response of the particular patient. The above dosages are exemplary of the average case, but there can be individual instances in which higher or lower dosages are merited, and such are within the scope of this invention.

The active agents of the present invention can be administered alone, or in admixture with a pharmaceutically acceptable carrier selected with regard to the intended route of administration and standard pharmaceutical practice. Pharmaceutical compositions for use in accordance with the present invention thus can be formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active agents into preparations that can be used pharmaceutically.

These pharmaceutical compositions can be manufactured in a conventional manner, e.g., by conventional mixing, dissolving, granulating, dragee-making, emulsifying, encapsulating, micronizing, entrapping, or lyophilizing processes. Proper formulation is dependent upon the route of administration

chosen. When a therapeutically effective amount of the active agents are administered orally, the composition typically is in the form of a tablet, capsule, powder, solution, or elixir. When administered in tablet form, the composition can additionally contain a solid carrier, such as a gelatin or an adjuvant. The tablet, capsule, and powder contain about 5% to about 95% of an active agent of the present invention, and preferably from about 25% to about 90% of an active agent of the present invention. When administered in liquid form, a liquid carrier, such as water, petroleum, or oils of animal or plant origin, can be added. The liquid form of the composition can further contain physiological saline solution, dextrose or other saccharide solutions, or glycols. When administered in liquid form, the composition contains about 0.5% to about 90% by weight of active agents, and preferably about 1% to about 50% of an active agents.

When a therapeutically effective amount of an active agent is administered by intravenous, cutaneous, or subcutaneous injection, the composition is in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred composition for intravenous, cutaneous, or subcutaneous injection typically contains, in addition to a compound of the present invention, an isotonic vehicle.

Suitable active agents can be readily combined with pharmaceutically acceptable carriers well-known in the art. Such carriers enable the active agents to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained by adding the active agents with a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients include, for example, fillers and cellulose preparations. If desired, disintegrating agents can be added.

The active agents can be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form, e.g., in ampules or in multidose containers, with an added preservative. The compositions can take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and can contain formulatory agents, such as suspending, stabilizing, and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active agent in water-soluble form. Additionally, suspensions of the active agents can be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils

or synthetic fatty acid esters. Aqueous injection suspensions can contain substances which increase the viscosity of the suspension. Optionally, the suspension also can contain suitable stabilizers or agents that increase the solubility of the compounds and allow for the preparation of highly concentrated solutions. Alternatively, a present composition can be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The active agents also can be formulated in rectal compositions, such as suppositories or retention enemas, e.g., containing conventional suppository bases. In addition to the formulations described previously, the active agents also can be formulated as a depot preparation. Such long-acting formulations can be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the active agents can be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

In particular, the active agents can be administered orally, buccally, or sublingually in the form of tablets containing excipients, such as starch or lactose, or in capsules or ovules, either alone or in admixture with excipients, or in the form of elixirs or suspensions containing flavoring or coloring agents. Such liquid preparations can be

prepared with pharmaceutically acceptable additives, such as suspending agents. An active agent also can be injected parenterally, for example, intravenously, intramuscularly, subcutaneously, intrathecally, intracisternally, or intracoronarily. For parenteral administration, the active agent is best used in the form of a sterile aqueous solution which can contain other substances, for example, salts, or monosaccharides, such as mannitol or glucose, to make the solution isotonic with blood.

For veterinary use, the active agents are administered as a suitably acceptable formulation in accordance with normal veterinary practice. The veterinarian can readily determine the dosing regimen and route of administration that is most appropriate for a particular animal.

I. *In Vitro* models

Example 1: Expression and Purification of Recombinant nNOS and PSD95 PDZ-Binding Regions

Construction of Recombinant GST-nNOS

Recombinant nNOS containing the PSD95 binding domains, but lacking the catalytic domain, was expressed in *E. coli* as a GST-fusion protein, and purified for use in the *in vitro* nNOS-PSD95 binding assay.

The coding sequence of human nNOS (accession No. D16408) was amplified from the human rat brain library (Clontech) according to the manufacturer's protocol, and subcloned into pCR2.1 for

sequencing. To construct the GST-nNOS plasmid, nucleotides 1-897 of the ORF (corresponding to amino acids (aa) 1-299) were amplified using the following primers:

Sense primer:

5' CCG AAT TCC ATG GAG GAT CAC ATG (SEQ ID NO: 26)

EcoRI aa 1

Antisense primer:

(CCG) 5' CTC GAG CCT AGG GGC TGC CAT TC

XhoI aa 299 (SEQ ID NO: 27)

The primers also served to introduce EcoRI and XhoI cloning sites into the 5' and 3' flanking regions respectively. After amplification, this sequence ("nNOS (1-299)") was subcloned using standard recombinant techniques into the EcoRI-XhoI sites of pGEX 4T3, such that the clone was in frame with the GST tag of the vector ("GST-nNOS").

Purification of GST-nNOS

Bacterial cultures expressing GST-nNOS were grown in LBM/Carb overnight at 37°C. The cultures were diluted 1:25 into 1 liter of LB medium (Luria-Butani medium)/50 µg/mL carbenicillin and grown at 37°C until a final OD_{600} of 0.6. The cultures then were induced with 0.2 mM IPTG (isopropyl β-D-thiogalactopyranoside) for 2 hrs (hours) at 37°C before the cells were harvested by centrifugation at 5000 rpm for 10 min (minutes) at 4°C. Bacterial cultures expressing GST-nNOS were harvested, and the

GST-protein purified according to the manufacturer's instruction (Pharmacia). Briefly, the bacterial cell pellet was lysed with calcium- and magnesium-free phosphate buffered saline (CMF-PBS), 0.5% TRITON® X-100 (50 mL per 1 liter of culture), and 50 µL of 100 mg/mL hen egg lysozyme (Sigma). After incubation on ice for 45 min, the lysate was sonicated 3 times for 10 sec (seconds), then centrifuged at 10,000 rpm for 15 min at 4°C. Soluble supernatant was applied to 50% glutathione SEPHAROSE® 4B (Pharmacia) bead slurry (1 mL per 1 liter of culture) previously blocked for 1 hr in 2% milk, 0.1% BSA (bovine serum albumin), 1x CMF-PBS and washed with CMF-PBS. After three bed volume washes using CMF-PBS/0.5% TRITON® X-100, the resin was washed again with CMF-PBS. nNOS (1-299) was eluted directly from the column by cleaving the protein off of GST resin with thrombin (50 units; Pharmacia) diluted in CMF-PBS. The resin was rotated for 4 hr at room temperature before eluate was collected. A single peak containing cleaved nNOS (1-299) was eluted and analyzed by SDS-polyacrylamide gel electrophoresis. This cleaved nNOS (1-299) then was used in the plate binding assay to measure the interaction between nNOS (1-299) and PSD95.

Construction of Recombinant PSD95 PDZ 1-3 Domains

PDZ domains 1-3 of PSD95 were used in the nNOS-PSD95 binding assay. To obtain the coding region, human PSD95 (accession no. NM_001365) was

amplified from a human brain library (Clontech) according to manufacturer's protocol, and subcloned into pCR2.1 for sequencing. Recombinant PSD95 (PDZ 1-3, aa 1-435) was PCR-amplified from pCR2.1/PSD95 using the following oligonucleotides:

Sense primer:
5'CCATGGAATACGAGGAAATCACATTG (SEQ ID NO: 28)
NcoI

Antisense primer:
5' AAGCTTCTACTGAGCAATGATCGTGAC (SEQ ID NO: 29)
HindIII

The PCR-amplified fragment again was cloned into pCR2.1, digested with NcoI and Hind-III, then ligated into similarly-digested pPinArab (referred to as Biotin expression plasmid in U.S. Patent No. 6,107,104, example 13) to make an in-frame fusion with the biotin acceptor peptide. The ligated vector and insert then were transformed into TOP10 cells (Stratagene, Torrey Pines, CA) and plasmid DNA was isolated with the QiAprep 8 miniprep kit (Qiagen, Thousand Oaks, CA).

Construction of pRSET-B Vector Containing Tat and 3E9 Sequences

Neuronal NOS (1-299) was subcloned into pRSET-B vector (Invitrogen, CA) with Tat, and 3E9 sequences inserted upstream to the open reading frame of nNOS (1-299). To generate this vector, the following oligonucleotides incorporating both Tat and 3E9 sequences were used:

Sense oligonucleotide:

5'CTAGCGGCTACGGCCGCAAGAAGCGCCGTCAACGCCGACGCGGCAAGCTG AAGCTGACCGGCGATGAAGCTGAATTCCTCGAGAGATCTGA (SEQ ID NO: 30)

Antisense oligonucleotide:

5'AGCTTCAGATCTCTCGAGGAATTCAGCTTCATCGCCGGTCAGCTTCAGCT TGCCGCGTCGGCGTTGACGGCGCTTCTTGCGGCCGTAGCCG (SEQ ID NO: 31)

The oligonucleotides were annealed by heating to 94°C for 3 min, and then slowly cooled to room temperature (one degree per min) to form an oligonucleotide double-stranded cassette. The cassette had 5' overhangs for subcloning into the pRSET-B vector. The resulting annealed oligonucleotides have the following (sense) sequence (SEQ ID NO: 32) that codes for the peptides or amino acids indicated below the nucleotide sequence. Restriction enzyme sites are indicated in italics:

5'CTAGCGGCTACGGCCGCAAGAAGCGCCGTCAACGCCGACGC GGCAAGCTG
NheI TAT peptide Gly
AAGCTGACCGGCGATGAAGCT GAATTCCTCGAGAGATCTGA
3E9 peptide *EcoRI* *XhoI* *Hind III*

The annealed oligonucleotides were serially diluted and ligated to pRSET-B previously digested with Nhe I and Hind III. The vector is referred to as pRSET/Tat-3E9.

Plasmid construction for Tat-nNOS (1-299)

The coding sequence for nNOS (1-299) was PCR-amplified using GST-nNOS (1-299) as a template with the following oligonucleotides:

Sense oligonucleotides with EcoRI in frame with 3E9:

5' CGGAATTCATGGAGGATCACATGT TCG G (SEQ ID NO: 33)

EcoRI M

Antisense oligonucleotides with Xho I site:

5' GGCCTCGAGCCTAGGGGCTGCCAT TCT TTG (SEQ ID NO: 34)

Xho I Stop

After amplification, the nNOS (1-299) sequence was subcloned using standard recombinant techniques into the EcoR I-Xho I sites of pRSET/Tat-3E9 vector, such that the clone was in frame with both Tat and 3E9 sequences.

Purification of Tat-nNOS (1-299)

Bacteria expressing pRSET/nNOS (1-299) (or Tat-nNOS) were grown in LBM/Carb overnight at 37°C. Cells then were diluted 1:250 into 1 liter of LB/-Carb and grown to an OD_{600} of 0.6. Cells were induced with 0.5 mM IPTG for 2 hrs at 37°C. Cells were harvested by centrifugation and lysed with 8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris, pH 8.0. Tat-nNOS was batch purified using Ni-NTA (nickel-nitriloacetic acid) affinity chromatography (Qiagen) at room temperature under denaturing conditions according to manufacturer's directions with modifications from N.A. Lissy et al. (1998, *Immunity*, 8:57-65).

After His-tagged proteins were bound to Ni-NTA resin, the resin was washed with the lysis buffer at pH 6.3 and 20 mM imidazole. This tagged Tat-nNOS then was eluted first with lysis buffer at pH 5.9 and 250 mM imidazole, then with the same buffer but at pH 4.5. Eluates containing the highest amount of Tat-nNOS then were collected, dialyzed at 4°C overnight against 1 x CMF-PBS, and stored at -20°C with 10% glycerol added.

Purification of biotinylated PSD95 (PDZ 1-3)

Bacterial cultures expressing PSD95 (PDZ 1-3) were grown in the presence of 4 μM biotin to allow endogenous biotinylation of proteins. Expression of PSD95 was induced with addition of 0.5% arabinose (overnight incubation at 30°C). The cells were harvested, resuspended in buffer A: 50 mM Tris, pH 8, 50 mM NaCl (sodium chloride), 2 mM EDTA (ethylenediaminetetraacetic acid), 4 mM DTT (11-dithiothreitol), and 10% glycerol. After lysing the cells with a FRENCH® press, the lysate was centrifuged at 48,000 x g for 45 min. The supernatant then was added to avidin resins (Pierce) which had been preblocked with 2 mM d-biotin in CMF-PBS, washed with 100 mM glycine, pH 2.8, and equilibrated with lysis buffer. After rotating the resins with the cell lysate for 4 hr at 4°C, the resins were spun down at 3600 rpm for 15 min at 4°C in a table-top centrifuge. The resins were washed to baseline with CMF-PBS. Biotinylated proteins were eluted by

incubation with 4 mL of buffer A and 5 mM biotin in CMF-PBS for 1 hr with rotation. To collect the eluate, the resin was spun at 3600 rpm for 15 min at 4°C. A second incubation was repeated, and the eluate again was collected by centrifugation. Both eluates containing biotinylated PSD95 (PDZ 1-3, >90% pure) were combined and dialyzed into CMF-PBS and concentrated using a Millipore CENTROCON® filter. Proteins were stored at -70°C in CMF-PBS and 10% glycerol.

Example 2: High Throughput Screening

A high throughput screen of a diverse chemical library was performed using an *in vitro* plate binding assay to identify inhibitors of the nNOS-PSD95 protein-protein interaction. The binding between the two proteins was quantified using time-delayed fluorescence (similar to that described in U.S. Patent No. 6,107,104).

nNOS (1-299) was diluted to 0.14 μM in CMF-PBS. nNOS (1-299) (50 μL/well) then was passively captured onto high binding IMMULON® 384 well plates and incubated overnight at 4°C. Excess protein was removed by repeated washing with CMF-PBS/-0.05% TWEEN® 20. The unbound sites on the plates then were blocked by adding 140 μL/well of SEA block (Pierce). The plates were incubated for one additional hour at room temperature before excess proteins were removed by repeated washing with CMF-PBS/0.05% TWEEN® 20. Biotinylated PSD95 (20 μL/well, 12.5 nM final) was added, and binding was continued

for 2 hrs at room temperature before the reaction was terminated by repeated washing with CMF-PBS/0.05 % TWEEN® 20. The biotinylated PSD95-nNOS (1-299) complex was detected using 50 µL of streptavidineuropium (diluted 1:1000 in assay buffer provided by Wallac). After further washing to remove nonspecifically bound proteins, 50 µL of enhancement solution (Wallac), diluted 1:1 in water, was added, and release of europium was measured by increased fluorescence using a DELFIA® research fluorometer (Wallac Victor reader).

nNOS and PSD95 bind to one another in a specific and saturable manner, with an estimated K_d of about 40-200 nM. Nonbiotinylated PSD95 competed with nNOS/biotinylated PSD95 binding with an IC_{50} of about 800 nM.

Compounds (158752 in number) from a chemical high throughput screen (HTS) library (each containing a pool of 22 compounds) in dimethyl sulfoxide (DMSO) were diluted to 18 µM in CMF-PBS/0.1% BSA, 10.05% TWEEN® 20, and 20 µL of the dilution was added to wells of a 384-well plate. To this 20 µL of pooled chemical library, 20 µL (25 nM) of biotinylated PSD95 was added. Test compounds were not preselected based on any known properties. When analyzing the results, a positive observation, or a "hit," was defined as a single compound capable of inhibiting 15% nNOS-PSD95 binding at a concentration of 9 µM or less. Percent binding was determined by measuring the amount of biotinylated PSD95-nNOS (1-299) complex in each well in the presence of pooled

compounds compared to the amount of biotinylated complex measured in the absence of any inhibitor.

Using these criteria, 32 pooled master wells were selected for deconvolution, based on the above criteria. At 22 compounds per well, a total of 704 compounds then were assayed individually at 40 μM for inhibition of nNOS-biotinylated PSD95 binding. From these assays, fourteen compounds were found to inhibit nNOS-PSD95 binding with an IC_{50} value between 26 nM-100 μM. Nine of these compounds then were tested in secondary *in vitro* and cell-based assays to identify PSD95-nNOS inhibitors that are specific, efficacious, and nontoxic.

Example 3: Cell-Based Efficacy Assays

Efficacy in Cell-Based Assay

Compound (1) also is effective in cell-based assays. In particular, compound (1) inhibited NMDA-induced guanosine 3',5'-cyclic monophosphate (cGMP) elevation in primary cultures of rat hippocampal neurons with an EC_{50} value of about 5 μM. In this test, primary neuronal cultures were preincubated with dimethyl sulfoxide (DMSO) or various concentrations of compound (1) for 15 minutes, then treated with a medium containing 5 μM sodium nitroprusside (SNP, an NO donor), or 100 μM NMDA for 15 minutes. cGMP was measured by radioimmunoassay (RIA) and the results are expressed as a percent of control (control is defined by NMDA-induced cGMP in cultures treated with DMSO). Toxicity was assessed

by measuring the levels of calcein (Molecular Probes) taken up by the neurons following a two-hour exposure to compound (1). As shown in Figure 3, compound (1) did not inhibit NO donor-induced cGMP elevation, indicating that molecule does not block pathways downstream of NMDA-activated nNOS. In addition, compound (1) is nontoxic up to 50 μM, which is ten times the allowable efficacy to toxicity ratio.

In an assay for cell-based efficacy, the inhibitors were expected to inhibit the NMDA receptor-PSD95-nNOS pathway. NMDA binds to NMDA receptors to activate nNOS, and thus NO production, in primary neuronal cells, resulting in neurotoxicity. NO production can be measured by increases in cGMP level (quantitated by RIA) in the cultured cells. Antisense PSD95 inhibited this NMDA-dependent pathway, suggesting that nNOS-PSD95 interaction is critical.

Neonatal rat hippocampi were cultured using the method of Brewer et al. (*J Neurosci Meth.*, *71*:143-58 (1997)). About 11 to 14 one-day-old neonatal rat hippocampi were dissected rapidly and placed in Hibernate A (Gibco-BRL) supplemented with B27 (Gibco-BRL) and 0.5 mM L-glutamine. Hippocampi were finely minced, then placed in Hibernate A containing 6 mg/mL papain (Sigma) and incubated at 37°C for 30 min with slow rotation. The tissues were washed gently three times in 5 mL of supplemented Hibernate A medium, then triturated three times in 2 mL of the same medium. The cell suspension was

collected and centrifuged at 1100 rpm for 2 min in a Beckman tabletop centrifuge. Supernatant was removed and the cell pellet gently resuspended in NEUROBASAL™-A medium (NBM-A, Gibco-BRL), supplemented with B-27, penicillin/streptomycin, L-glutamine, and 5 µg/mL basic FGF (Gibco-BRL). Cells were plated at 2 x 10^5 cell/mL in 24-well plates (Corning), previously coated for 16-24 hr with 1 mg/mL poly D-lysine (Sigma) in water. Two days after plating, the cells were treated with 5 µM ara-C (Sigma) for 2-3 days before feeding with fresh supplemented NBM-A. Cells were cultured for 14-21 days, with medium changed every 5-7 days, before use in cell-based assays.

Inhibitors of nNOS-PSD95 binding were tested on these primary rat hippocampal cultures for their ability to disrupt the NMDA increase in cGMP production. Fourteen- to twenty-one-day-old neuronal cultures were washed gently with warm control saline solution (CSS, 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM Tris-HCl, and 15 mM glucose) and incubated in 250 µL of CSS at 37°C for 15 min. Compounds were made as 4 x effective stock solutions of CSS containing 200 µM of isobutylmethylxanthine (IBMX, Sigma). An equivalent volume of DMSO was used as vehicle control. Compounds were added to the neuronal cultures and incubated for 15 min at 37°C. NMDA (Sigma, 100 µM final concentration) then was added and the neurons incubated at 37°C for a further 5-15 min before washing in warmed 1 x Hanks's balanced saline solution (HBSS). Ice-cold

ethanol was added to extract cyclic nucleotides. The ethanol extract was dried by Speedvac. cGMP levels in the samples were determined by radio-immunoassay (RIA kit purchased from Perkin Elmer/New England Nuclear, MA) according to manufacturer's instructions.

Toxicity of the compounds also was assessed using neuronal cultures. The cultures were incubated in the presence of compounds or vehicle as described above. Cultures then were gently washed twice with 1 x HBSS and incubated in 2 μM calcein (Molecular Probes) in 1 x HBSS at 37°C for 30 min. The cultures were washed twice with 1 x HBSS and calcein fluorescence measured on a Cytofluor 2350 (Millipore). Calcein fluorescence is a direct measure of cell viability.

Five of the nine inhibitors identified in the high throughput screen (see Example 2) inhibited an NMDA-dependent increase in cGMP with EC_{50} in the 3-20 μM range, and an efficacy/toxicity window greater than 5-fold.

As discussed hereafter, compound (1) inhibited PSD95-nNOS *in vitro*, and also inhibited NMDA-induced cGMP elevation in primary rat hippocampal cultures with an EC_{50} of about 5 μM. Compound (1) did not inhibit cGMP production induced by the NO-donor sodium nitroprusside, suggesting that compound (1) does not block pathways downstream of NMDA-activated nNOS. Compound (1) also was found to be nontoxic up to 50 μM.

Whereas compound (1) lost some of its inhibitory activity in the presence of 50% serum when assayed for effect on NMDA-induced cGMP elevation, the EC_{50} for compounds (2) and (3) were not significantly changed.

Example 4: Organotypic Hippocampal Slice Culture

The NMDA receptor-PSD95-nNOS pathway also is important for hypoxia-induced damage in neurons. Organotypic culture thus was used to test whether inhibitors of nNOS-PSD95 in blocking this pathway would also prevent hypoxia-induced cell death. Organotypic hippocampal slices were cultured as described in (L. Stoppini et al., *J. Neurosci. Meth.*, *37*:173-82 (1991)). Briefly, cultures were prepared from day 5 to 7 neonatal rats (Sprague-Dawley, Bantin and Kingman Inc., Fremont, CA). Slice cultures were grown on sterile transparent ANOCEL® membranes (Millipore) for 12-14 days before experimentation in 50% MEM (Gibco-BRL) supplemented with HEPES and sodium bicarbonate, 25% HBSS, 25% horse serum, and glucose to a final concentration of 6.5 ng/mL The cultures were grown at 36°C, 90-100% humidity, and 5% CO_2.

For exposure to hypoxic and hypoglycemic conditions presumed to approximate conditions of ischemic insult, cultures were placed in glucose-free HBSS. Compounds were added to the medium as small aliquots in glucose-free HBSS and the cultures then were transferred into an anaerobic chamber, pre-equilibrated at 37°C and with an atmosphere of

10% H_2, 5% CO_2 and 85% N_2. Exposure time was 25 to 30 min. Cultures were removed from the anaerobic chamber and transferred back to prewarmed medium containing compound or vehicle in the same concentration used for the experiment, and returned to the incubator. Cultures were incubated for 16 to 24 hrs before measuring neuronal death within each slice.

Neuroprotection was calculated as the percentage of dead cells in test compound-treated slices relative to DMSO-treated slices. The aim was to obtain 50% to 60% cell death in the control slices as determined by propidium iodide staining. MK-801, an NMDA receptor antagonist, was used as a positive control. Consistent with previous studies, MK-801 at 10 μM inhibited >90% of the ischemia-induced cell death.

Figure 4 illustrates that compound (1) significantly reduced NMDA-induced neurotoxicity in this organotypic slice culture assay. (Each bar in Figure 4 represents 11 slice cultures.) Treatment of the cultures with compound (1) at 0.5 and 1 μM inhibited >90% of ischemia-induced cell death. The extent of neuroprotection was comparable to that observed with MK-801-treated cultures. It is believed that disrupting nNOS-PSD95 interaction allows for uncoupling of NMDA activation of nNOS, and that this mechanism assists in protecting against ischemia-induced cell death.

Example 5: nNOS Enzymatic Assays

Inhibitors of PSD95-nNOS protein-protein interaction should not affect the catalytic activity of nNOS. Indeed, the nNOS protein used in the binding assay contains only the PDZ-binding domains, and does not contain the catalytic domain. Because NOS enzymatic function is critical for many cellular functions, it is preferred that inhibitors of PSD95-nNOS have little to no effect on NOS catalytic activity, in order to avoid impairing the function of other NOS enzymes, e.g., eNOS.

To test whether nNOS targeting inhibitors have any effect on the catalytic activity of NOS, a NOS enzymatic assay, based on (J. Dawson et al., *Meth. Mol. Biol.*, *100*:237-42 (1998)), was used.

Isolation of nNOS from Rat Brain

Rat brain nNOS was purified using a combination of adenosine 2',5'-bisphosphate (2',5'-ADP)-Sepharose and calmodulin affinity chromatography (modification of H.H. Schmidt et al., *Proc. Nat'l. Acad. Sci. (USA)*, *88*:365-9 (1991)). All purification was carried out at 4°C, in the presence of protease inhibitors. Briefly, frozen rat brains (Pel-Freez, Rogers, AR) were thawed and homogenized with polytron on ice in 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA and 10% glycerol (5 mL/g tissue). After centrifugation at 10,000 x g for 20 min, the supernatant was collected and further centrifuged at 100,000 x g for 60 min. The

resultant supernatant was incubated with 1-2 mL of 2',5'-ADP Sepharose pre-equilibrated with homogenization buffer for 30 min. The mixture then was poured into a column, and flow through was collected. Nonspecifically bound proteins were removed with 25 bed volumes of homogenization buffer containing 0.5 M NaCl. The column was washed again with five bed volumes of homogenization buffer before proteins were eluted with four bed volumes of homogenization buffer and 10 mM NADPH. Peak protein fractions were collected, and concentrated prior to storage.

nNOS Activity Assays

Neuronal NOS enzymatic activity assays were performed essentially as described by J. Dawson et al., *supra*, with the exception that the final assay concentration of HEPES was 50 mM instead of 100 mM, and the reference inhibitor stocks (N^5-[imino(methylamino)methyl]-L-ornithine (L-NMMA) and N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) were dissolved in 25% DMSO/50mM HEPES, pH 7.4. All buffers, inhibitors, plates, and equipment were prewarmed to 37°C prior to assaying. In brief, 200 µL of 1.25 x assay buffer (consisting of HEPES, pH 7.4, DTT, $CaCl_2$, oxyhemoglobin, cofactor cocktail (NADPH, FMN, FAD, calmodulin) and BH4 ((6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride) was combined with 20 µL of 12.5 x inhibitor (or vehicle control) and 10 µL of 25 x L-arginine (a substrate for nNOS) in a prewarmed 96-well plate. The assay was

initiated by addition of 20 µL of 12.5 x partially purified rat brain nNOS (previously diluted into 50 mM HEPES, pH 7.4). After mixing the reaction for 15 sec, continual measurements at 405 nm and 420 nm were collected for 30-60 min at the shortest interval possible (usually 10-20 sec) on a SpectraMax 250 (Molecular Devices). Final Assay Concentration: 50mM HEPES, pH 7.4, 100 µM DTT, 1 mM $CaCl_2$, 5 µM oxyhemoglobin, 12 µM BH4, 120 µM NADPH, 1 µM FMN, 1 µM FAD, 0.1 µM calmodulin, and 2 µM L-arginine (for inhibition studies).

Compound (1) and a comparative compound (1) were tested in an nNOS catalytic assay and each found to have no effect up to 100 µM. Comparative compound (1) has the structure

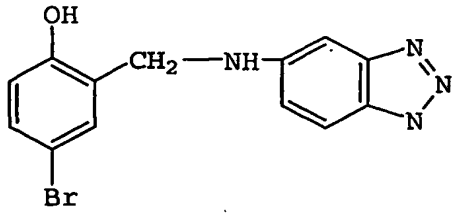


Figure 5 shows that compound (1) did not inhibit the catalytic activity of nNOS, even at 200 µM. L-NMMA, an arginine analog and a nonselective nNOS inhibitor having an IC_{50} of 1 µM, was used as a reference inhibitor.

II. Animal models

Inhibitors of the nNOS-PSD95 interaction are expected to disrupt the NMDA receptor-PSD95-nNOS

pathways. To validate these inhibitors in animal models, the small molecule inhibitors and Tat-nNOS were tested on various animal pain models where the NMDA receptor-PSD95-nNOS pathway has been shown to be important.

Example 6: Acute Pain

(a) "Tail-flick" Model

It was found that compound (1) fully reversed thermal hyperalgesia induced by NMDA in a mouse tail-flick model described in C.A. Fairbanks, *Proc. Nat'l. Acad. Sci. (USA)*, *97*:10584-9 (2000). NMDA delivered intrathecally to ICR mice induces a thermal hypersensitivity, which may be measured by tail immersion in warm water (49°C). This NMDA-induced hypersensitivity or hyperalgesia can be blocked by inhibitors of nitric oxide synthase (K.F. Kitto, (1992), *supra*). To measure antinociception with tail immersion, the latency to rapid tail flick before and during NMDA administration was measured. Antinociceptive effect was calculated as percent maximum possible effect by the formula: (postdrug latency-predrug latency)/(12-predrug latency) x 100%. Experiments were performed under conditions where the control or tested inhibitors were blinded. Eight to ten mice were used for each dose point, and data were calculated as mean ± SEM. Compound (1) initially was dissolved in 5% DMSO and 1 mole equivalent of NaOH, then diluted with 5% DMSO in saline.

The corresponding vehicle control used the same diluent.

Compound (1) and NMDA were coadministered intrathecally (i.t.) into mice, and tail flick latency to warm water was measured before and 5 min. after administration. NMDA administered i.t. into mice induces a thermal hypersensitivity, which requires the activation of NOS.

Compound (1) dose-dependently and fully reversed thermal hyperalgesia (hypersensitivity) induced by NMDA. The EC_{50} was <0.03 fmole/mouse (Fig. 6). NOS catalytic enzyme inhibitors, such as L-NAME or 7-Ni, inhibited NMDA-induced hyperalgesia with EC_{50} about 1 nmole/mouse. Compound (1), at higher concentrations (1-300 nmole/mouse), prolonged the thermal latency of tail flick to about 200% of the latency of naïve mice. This demonstrates that compound (1) also can be analgesic at higher concentrations.

(b) NMDA-Dependent, nNOS-Independent Scratching and Biting Behavior

To test for potential nonspecific side effects on NMDA receptors that are unrelated to inhibition of nNOS-PSD95 interaction, the effect of nNOS targeting inhibitors on NMDA-induced nociceptive behavior and motor impairment also were measured. NMDA delivered intrathecally to ICR mice produces scratching and biting behavior directed to the hind limbs, which is antagonized by NMDA receptor antagonists, but not L-NAME or 7-Ni (Fairbanks

et al., *supra*). After NMDA administration, the number of hind limb-directed behaviors elicited within one minute was measured. NMDA receptor antagonists, such as MK801 (Fairbanks et al., *supra*), inhibited this response, while NOS catalytic inhibitors, such as L-NAME, had no effect. Rotarod assay was also used on the same mice (Fairbanks et al., *supra*). After two training sessions, mice walked for 300 sec on an accelerating rotarod. The latencies to fall before and after delivery of vehicle or NMDA with or without inhibitor were compared. NMDA-induced motor impairment is dependent on the NMDA receptor, but not on nNOS.

In order to separate an effect of a test compound on NMDA receptor-requiring action from effects on an action requiring nNOS, NMDA-induced nociceptive behavior, motor impairment, and thermal hypersensitivity were measured in the same animal and in the same experiment after intrathecal administration of NMDA and targeting inhibitors. Compound (1) had no effect on the NMDA-induced itching/scratching behavior or NMDA-induced motor impairment, consistent with effects seen by nNOS inhibitors, such as 7-Ni.

Example 7: Inflammatory pain model

Formalin injected subplantarly induced two phases of paw licking behavior that are considered models of acute and inflammatory pain, respectively. Using this model, compound (1), in PBS and 5% DMSO, was injected i.t. into mice. Immediately or 30

minutes after administration of the respective test substances, formalin (5% solution) was injected subplantarly in a volume of 0.02 mL. The induced hind paw licking time was recorded during the first inflammatory phase (0-5 min) and second inflammatory phase (20-30 min) periods after formalin challenge. Compound (1), at 0.3 nmole/mouse, inhibited 50% of the second inflammatory phase (n=4 per dose).

Example 8: Chronic visceral pain model

Visceral hypersensitivity, a subset of chronic neuropathic pain, can be studied in animals as a model for irritable bowel syndrome. It can be induced by intracolonic administration of 0.6% acetic acid (B. Greenwood van Meerveld, unpublished data). One to four weeks after the acetic acid priming (or saline pretreatment), intracolonic injection of capsaicin was used as a stimulus. In animals that have been pretreated with saline, capsaicin (0.6% solution) will evoke 40-50 scratching and biting behaviors directed to the perineum over a 20 min period. Animals that have been pretreated with a single injection of 0.6% acetic acid will respond with 110-150 scratching and biting behaviors in a 20 min period. Because of the duration of the effect, this can be interpreted as a chronic visceral hypersensitivity model. This hypersensitivity is dose-dependently reversed by intrathecally administered morphine, clonidine, MK 801, and 7-Ni, suggesting that NMDA receptor-nNOS pathway is involved. Figure 7 illustrates the effect of compound (1) on

this model of chronic visceral hypersensitivity. The results were calculated as mean ± SEM with 6 mice used per dose point. Compound (1) dose-dependently and fully reversed the visceral hypersensitivity upon capsaicin challenge in animals treated with acetic acid one week prior to the challenge. The EC_{50} was about 0.005 nmole/mouse.

Example 9: Chronic Pain Rat Model

To test the efficacy of nNOS targeting inhibitors in chronic neuropathic pain model, the rat model of G.J. Bennett et al., *Pain, 33*:87-107 (1988) was used. The Bennett et al. procedure induces a painful peripheral mononeuropathy produced by chronic constriction injury (CCI) of the sciatic nerve, and then measures alterations of behavior in relation to putative analgesic treatments. This CCI model, often referred to as the Bennett model, was the first animal model to demonstrate that NMDA receptor antagonists relieve neuropathic pain, and currently is the standard model used to validate efficacy of preclinical candidates in neuropathic pain.

IT and Neuropathic Surgeries

Adult male rats (350-450g) were anesthetized intraperitoneally with ketamine/medatomidine. The animals underwent two procedures. The first was implantation of a 17 cm catheter into the spinal subarachnoid space using the method described in

T.L. Yaksh et al., *Science, 192:*1357-8 (1976). Then, loose ligation was performed on the left sciatic nerve as described by Bennett et al. These CCI rats were then observed for spontaneous pain behaviors (paw-protecting postures), allodynia (a condition in which ordinary, nonpainful stimuli evoke pain), mechanical and thermal hypersensitivity, and reduced grooming. Indicators of neuropathic pain emerged gradually over the first 1-7 days following the lesion, and persisted for up to 2 months.

Behavioral Testing/Drug Testing

Mechanical allodynia was measured using von Frey hairs. The animals were tested presurgery, and every 1 or 2 days for up to 14 postoperative days or until neuropathic pain developed. Neuropathic pain occurred between postoperative days 1-7. During routine testing, an animal was allowed to acclimate to the surroundings for 20 minutes. Once acclimated, von Frey hairs of increasing force were applied to the paw and held for 8 seconds or until the animal withdrew its paw. Each animal was tested every 5 minutes, alternating between left and right paw. Increasing hair sizes were used until paw withdrawal was observed and the value of von Frey hair was recorded.

Once there was a right-left-paw score difference of at least 2 hair sizes, the animal was drug tested. Drug or vehicle was administered intrathecally and each animal was tested immediately

after injection. Behavioral testing continued at 5-minute intervals for at least 2 hours, and further if necessary until the responses returned to baseline. Rats were tested using 15.5 µg (50 nmole)/10 uL (n=3) or 30.9 µg (100 nmole)/10 uL (n=4) of compound (1) or vehicle (n=5). After injection, the catheter was flushed with 10 uL saline. All rats were tested the following day for one hour with 15 µg/10 uL of morphine to ensure proper placement of the catheter.

Histology

Following morphine administration, the animals were given a lethal dose of pentobarbital (50 mg, i.p.) and transcardially perfused with formalin. A saturated solution of Fast Green dye (10 uL) then was injected into the catheters followed by 10 uL saline. The spinal cord was exposed and the position of the catheter tip and the location of the dye were noted. The ideal location of the catheter tip is where the thoracic and lumbar segments meet. Animals in which the catheter tip deviated from this location by more than about 1 cm or were unresponsive to i.t. morphine were excluded from the study.

Effect on Neuropathic Pain

CCI rats showed a marked increase in mechanical sensitivity in the injured paw (see Fig. 8 for effect of vehicle control on left injured and right uninjured paws). The rats were reluctant to

put weight on the paw and often maintained the paw in a guarded position. Compound (1) was administered i.t. in 20% DMSO in PBS. A low dose (15.5 µg/10 uL, n=6) of compound (1) showed a small, but significant, effect on the injured paw, which consisted of a higher threshold in mechanical sensitivity (1-2 hair sizes above baseline, see Figure 9). There was no difference in sensitivity on the uninjured side (n=3). A higher dose (30.92 µg/10 uL, n=6) of compound (1) completely normalized the behavior response of CCI rats, with increases of 3-4 hair sizes on the injured side. Again, no effect on the uninjured side was observed (n=5, data not shown). The onset of this effect was rapid, and the duration of the effect was about 2.5-3 hours. See Fig. 9. During the peak drug effect, several animals did not hesitate to put weight on the foot and did not maintain the foot in a guarded position. The effect was not attributed to a local anesthetic action of compound (1), which would cause numbness on the injured foot, and interfere with their ability to walk on the injured side.

As a further control, comparative compound (1), an inactive analog of compound (1), was administered in 40% DMSO in PBS. Comparative compound (1) had no effect on nNOS-PSD95 binding or in cell based assay, was ineffective compared to vehicle control in this model (n=6, data not shown).

Additional tests have shown that compounds of general structural formula (I) are particularly effective disrupting agents of PSD95-nNOS inter-

action *in vitro*. For example, Figure 2 shows that compound (1) has an IC_{50} value of 55 μM.

Example 10: Cell Permeability Assay

In another test, compound (1) was found to permeate a cell, as assessed by the Madin Darby Canine Kidney (MDCK) assay.. (J.D. Irvine et al., *J. Pharm. Sci.*, *88*:28-33 (1999)). MDCK monolayers were grown to confluence and equilibrated in HBSS with 2% DMSO (transport medium). The electrical resistance of each monolayer was measured using an STX-2 "chop-stick" electrode and volt-ohm meter to ensure the integrity of the monolayers and tight junction development. Transport assay donor solutions consisted of 200 μM of compound (1) in transport medium. An aliquot from the receiver compartment was assayed at 1 and 3 hours. An aliquot from the donor compartment was assayed at the three-hour timepoint. Samples were analyzed and quantified by HPLC with detection at a wavelength of 200 nm using a Luna (Phenomenex 5 μm, ODS(2), 150 x 4.6 mm) column and a CH_3CN (0.05% RFA)/H_2O (0.05% TFA) gradient. Each determination was performed in triplicate. Compound (1) had a high transport rate across MDCK cell monolayers, with an apparent permeability of $6.3 \pm 1.5 \times 10^{-6}$ cm/s in MDCK cells. This is comparable to that of timolol which was used as a positive control.

Example 11: Tat-NOS Inhibits PSD95-nNOS Binding and is Efficacious in Cell-Based Assays and Animal Pain Models

In other tests, it was shown that the Tat-nNOS fusion protein (1-299) gave similar results to compound (1). Tat-nNOS(1-299) contains a region that is critical to binding PSD95, but does not contain the catalytic domain. This fusion protein is envisioned to enter cells and displace the wild type catalytically active nNOS, thus acting as a nNOS targeting inhibitor. Figure 10 shows that the Tat-nNOS fusion protein inhibited PSD95-nNOS *in vitro* binding with an IC_{50} of about 0.2 μM. In addition, primary hippocampal neuron cultures were incubated with various concentrations of purified Tat-nNOS for 30 minutes prior to treatment with 100 μM NMDA for 15 minutes. cGMP levels were measured by RIA. As can be seen in Figure 11, Tat-nNOS inhibited NMDA-induced cGMP elevation in primary hippocampal cultures with an EC_{50} of 500 nM, similar to that of compound (1). Finally, Fig. 12 shows that Tat-nNOS fusion protein also fully reversed the thermal hyperalgesia induced by NMDA with an EC_{50} of 0.2 fmole/mouse and at higher concentrations (>10 fmoles/mouse) showed analgesic effects similar to those found for compound (1). Tat-nNOS tested (at 0.225, 2.25, and 22.5 fmole/mouse) in the absence of NMDA, also showed analgesic activity, illustrating that nNOS targeting disruptors also can be analgesics.

Tat-nNOS was also tested in the Bennett Chronic pain rat model described in Example 9. Tat-nNOS (at 7 nmole/rat) reversed the mechanical allodynia associated with neuropathic pain (Fig 13). The onset of reversal was fast and the effect lasted for 150 min.

Example 12: Side Effect Profile of Inhibitors of nNOS-PSD95 Interactions

NMDA receptor antagonists have a narrow efficacy to toxicity ratio. Side effects of NMDA receptor antagonists can be measured by motor impairment in mice using the rotarod measurement. Compound (1) was administered i.t. into naïve mice (in the absence of NMDA), and the amount of time the mice remained on the rotarod was measured. The ability of the compound to change "normal pain sensation" i.e., exhibit analgesia, also was measured in the same mice using tail flick. Compound (1) was found to impair motor movement only at the highest concentrations of greater than 3 pmole/mouse. At that dose, compound (1) also blocked "normal" pain sensation, which can be attributed to a motor impairment because the two dose responses overlap (see Fig. 14). The concentration of compound (1) required to inhibit motor movement is much greater than the concentration required to inhibit NMDA-induced hypersensitivity, as shown in Fig. 14. Thus, the efficacy of compound (1) (i.e., antihyperalgesia) compared to adverse side effects

(i.e., motor impairment) is at least a few hundred fold.

More particularly, in Fig. 14, compound (1) or MK801 (NMDA receptor antagonist) was administered i.t. into naïve mice. Tail flick latency (in naïve mouse-analgesia, ▼ or in NMDA induced hypersensitivity, ■) and motor impairment (●) were measured 5 minutes after compound administration.

Example 13: Systemic Administration of Disruptors of nNOS-PSD95 Interaction

To determine whether compound (1) can cross the blood brain barrier, compound (1) was administered systemically. In this test, compound (1) was administered intraperitoneally (i.p.), then NMDA was administered i.t. after one hour. Tail flick latency was measured 5 minutes after NMDA administration. Intraperitoneal administration of compound (1) in mice fully and dose-dependently reversed NMDA induced hypersensitivity (Fig. 15). When motor impairment was measured in the same mice, compound (1) had only a small effect on motor movement at the highest dose tested.

In Fig. 15, compound (1) was administered i.p. into mice. One hour after administration of compound (1), NMDA was administered i.t. to induce hypersensitivity (■) as measured by tail flick latency in warm water. After tail flick latency was measured, motor impairment (●) was measured.

Example 14: Efficacy of a PSD95-nNOS Interaction Disruptor Over Time

To measure the duration of efficacy of compound (1) after i.p. or i.t. administration, compound (1) was administered i.p. or i.t. for various times before i.t. administration of NMDA. Tail flick latency was measured 5 minutes after i.t. administration of NMDA. The effects of compound (1) were observed after 30 minutes of i.p. administration, whereas the effects of i.t. administration were observed much more quickly. The effects of both i.p. and i.t. administration lasted for more than 100 minutes. Vehicle control had no effect.

In Fig. 16, compound (1) was administered either i.t. (o, 1 pmole/mouse) or i.p. (■, 1 mg/kg) into mice for the time indicated. NMDA then was administered i.t., and tail flick latency measured after 5 minutes.

Overall, Examples 12-14 show the systemic efficacy of a PSD95-nNOS interaction disruptor, as exemplified by compound (1). These experiments were designed to determine whether compound (1) crosses the blood-brain barrier by using the pain model as a bioassay. It was found that i.p. administration of compound (1) potently reversed NMDA-induced hyperalgesia as measured by tail flick latency, by exhibiting an ED_{50} of about 0.1 mg/kg, with full efficacy at 1 mg/kg with minimal motor impairment (as measured by rotarod). At 1 mg/kg, efficacy was observed as early as 30 minutes after administration and lasted for over 150 minutes. Similar results

were observed for the i.t. administration of compound (1), but with an earlier onset of efficacy. In particular, efficacy was observed at the first time point measured, peaking at about 20 minutes, and beginning to decline at 110 minutes. Efficacy still was observed at 190 minutes. In particular, the test results show that compound (1) can cross the blood-brain barrier after systemic administration, and that no overt toxicity was observed at a full efficacy dose of 1 mg/kg.

Modifications and variations of the invention as hereinbefore set forth can be made without departing from the spirit and scope thereof, and, therefore, only such limitations should be imposed as are indicated by the appended claims.

WHAT IS CLAIMED IS:

1. A method of alleviating pain or a sensation thereof comprising administering to a mammal in need thereof a therapeutically effective amount of an agent that disrupts an interaction between neuronal nitric oxide synthase (nNOS) and Post Synaptic Density Protein 95 (PSD95) on a protein related thereto.

2. The method of claim 1 wherein the protein related to PSD95 is selected from the group consisting of PSD93/chapsyn-110, SAP97/hdlg, and SAP102.

3. The method of claim 1 wherein the pain comprises acute pain.

4. The method of claim 1 wherein the pain comprises chronic pain.

5. The method of claim 1 wherein the pain comprises nociceptive pain.

6. The method of claim 1 wherein the pain comprises neuropathic pain.

7. The method of claim 6 wherein the neuropathic pain is selected from the group consisting of glossopharyngeal neuralgia, postherpetic neuralgia, central pain caused by a spinal cord injury, stump pain, phantom limb pain, neuropathic cancer pain, limb pain after traumatic nerve injury, peripheral neuropathy, complex regional pain disorder, arachnoiditis, carpal tunnel syndrome, a nerve root disorder, nerve root compression, nerve root damage, and surgery-induced nerve injury.

8. The method of claim 1 wherein the pain comprises inflammatory pain.

9. The method of claim 8 wherein the inflammatory pain comprises soft tissue pain, joint pain, bone pain, postoperative pain, osteoarthritis pain, rheumatoid arthritis pain, muscle pain, tendon pain, ligament pain, traumatic injury pain, sports or exercise injury pain, an inflammatory myopathy pain, muscle cramp pain, myalgia of neurogenic origin, drug-induced myalgia, myalgic encephalomyelitis, chronic back pain, upper extremity pain, and fibromyalgia.

10. The method of claim 1 wherein the pain comprises visceral pain.

11. The method of claim 10 wherein the visceral pain is attributed to acute appendicitis, mesenteric lymphadenitis, Crohn's disease, a tubo-ovarian disorder, a renal disorder, acute pancreatitis, chronic pancreatitis, peritonitis, acquired immunodeficiency syndrome, an intestinal obstruction, opiate withdrawal, chronic abdominal pain, an atypical gastro-esophageal reflux disorder, a gastric ulcer, irritable bowel syndrome, or constipation.

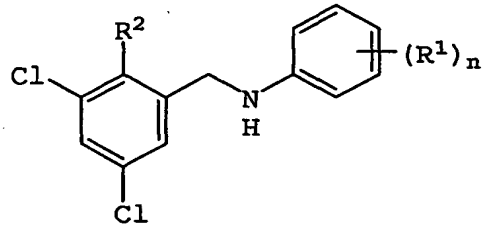
12. The method of claim 1 wherein the pain comprises a pain attributed to chronic arthritis, acute arthritis, a chronic back condition, an acute back condition, a cancer, a cancer treatment, a failed back syndrome, fibromyalgia, herpes zoster, intercostal pain, myofascial pain dysfunction syndrome, a neuralgia, a neuropathy, occipital neuralgia, phantom limb syndrome, postherpetic neuralgia, reflex sympathetic dystrophy, carpal tunnel syndrome, sciatica, trigeminal neuralgia, reflex sympathetic dystrophy, causalgia, degenerative joint disease, spinal stenosis, fibrositis, a trigger point, diabetic neuropathy, disc disease, amputation, radiculopathy, arachnoiditis, labor (of childbirth), a central nervous system disorder, headache, migraine headache, headache associated with head trauma, headache associated with a vascular disorder, headache associated with substance withdrawal, or headache associated with a metabolic disorder.

13. The method of claim 1 wherein the pain is back pain, phantom limb pain, postoperative pain, thoracic pain, sacroiliac joint pain, post-trauma pain, facial pain, trigeminal pain, neck pain, arm pain, shoulder pain, musculoskeletal pain, complex regional pain, labor pain, oral pain, oro-facial pain, dental pain, periodontal pain, gingival pain, mucosal pain, eye pain, ear pain, heart pain, vascular pain, haemopathic pain, chronic pelvic pain, bladder pain, dysuria, obstetric pain, and genitourinary pain.

14. The method of claim 1 wherein the disrupting agent is specific to the PSD95-nNOS interaction.

15. The method of claim 1 wherein the disrupting agent is selected from the group consisting of

(a) a compound having a general structural formula



wherein R^1, independently, is selected from the group consisting of C_{1-4}alkyl, halo, CF_3, OCF_3, $C(=O)R^a$, $C(=O)OR^a$, $N(R^a)_2$, $C(=O)N(R^a)_2$, $NR^aC(=O)N(R^a)_2$, OR^a, SR^a, NO_2, CN, $SO_2N(R^a)_2$, SOR^a, SO_2R^a, and OSO_2CF_3,

R^2 is hydro or OH,

R^a, independently, is selected from the group consisting of hydro, C_{1-4}alkyl, aryl, and heteroaryl, and

n is an integer 0 through 4,

wherein two R^1 groups can be taken together with the carbon atoms to which they are attached to form an optionally substituted 5- to 7-membered aliphatic or aromatic ring, and optionally containing one to three heteroatoms selected from the group consisting of oxygen, nitrogen, and sulfur,

or a pharmaceutically acceptable salt, solvate, or prodrug thereof;

(b) a natural product extract from a fungal or microbial source;

(c) a peptide having a sequence

A-B-C-D-E,

wherein A is null, Pro, or Val, having a terminal NH_2 group optionally acetylated or linked to Tat;

B is Glu, Gln, or Arg;

C is Thr;

D is Asp, Asn, or His;

E is Val, Leu, or Ile having a terminal $-CO_2H$ group,

and wherein B can be Asp, if D is Glu;

(d) Tat-nNOS (1-299); and

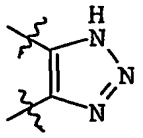
(e) mixtures thereof.

16. The method of claim 15 wherein the compound has a general structural formula wherein n is 0 to 3; R^1 is selected from the group consisting of halo, OR^a, $C(=O)OR^a$, C_{1-4}alkyl, NO_2, and $N(R^a)_2$; and R^2 is OH.

17. The method of claim 16 wherein R^1 is selected from the group consisting of chloro, fluoro, OH, $C(=O)OCH_3$, $C(=O)OH$, C_{1-4}alkyl, NO_2, and NH_2.

18. The method of claim 16 wherein the compound has a general structural formula wherein two R^1 groups are taken together to form a 5- or 6-membered heteroaryl group.

19. The method of claim 18 wherein the heteroaryl group is selected from the group consisting of



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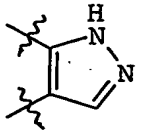

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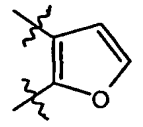

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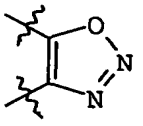

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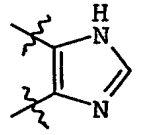

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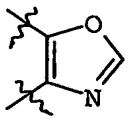

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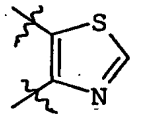

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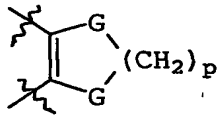
20. The method of claim 15 wherein the compound has a general structural formula wherein two R^1 groups are taken together, with the phenyl ring to which they are attached, to form naphthalene, indene, benzoxazole, benzothiazole, benzisoxazole, benzimidazole, quinoline, indole, benzothiophene, or benzofuran;

or are taken together to form



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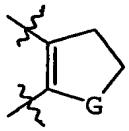

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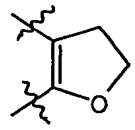
wherein p is 1 or 2, and G, independently, is $C(R^a)_2$, O, S, or NR^a.

21. The method of claim 20 wherein two R^1 groups are taken together to form



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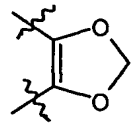

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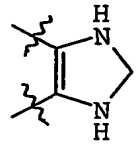

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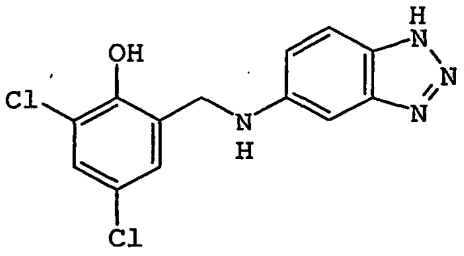
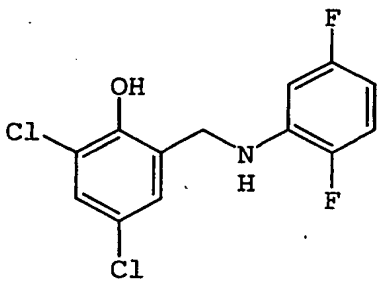
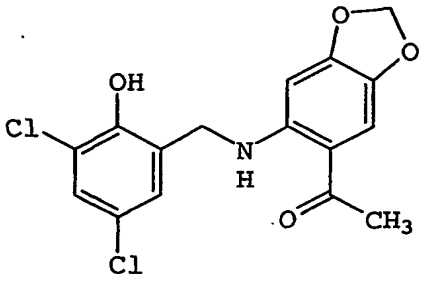
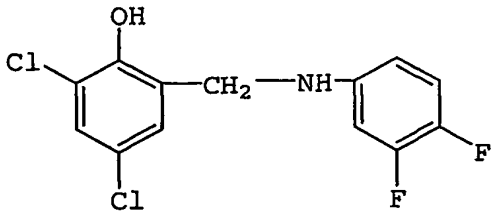
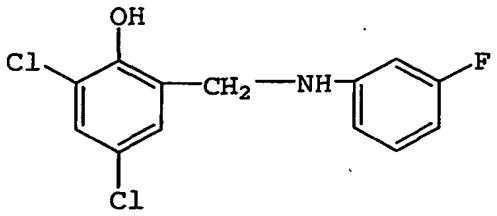
22. The method of claim 15 wherein the compound is selected from the group consisting of

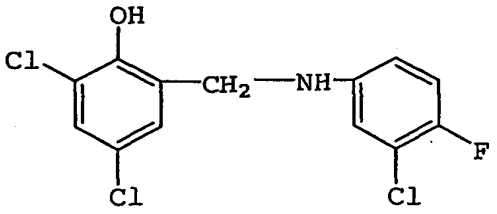


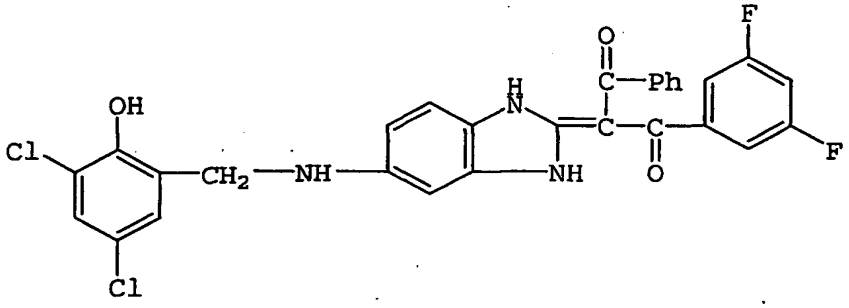


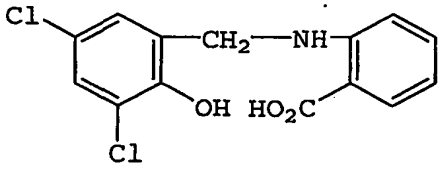


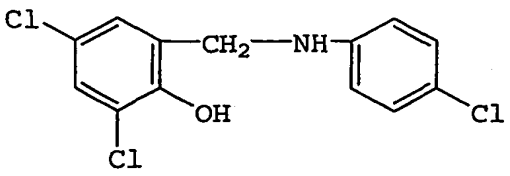


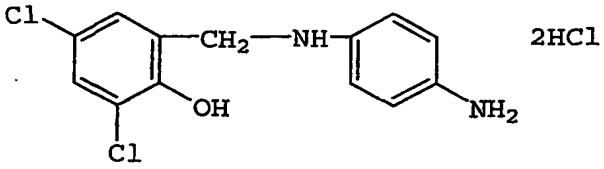


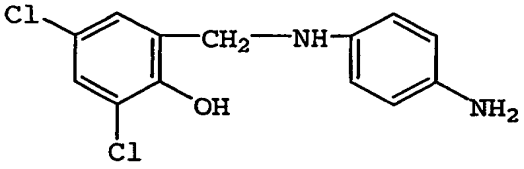


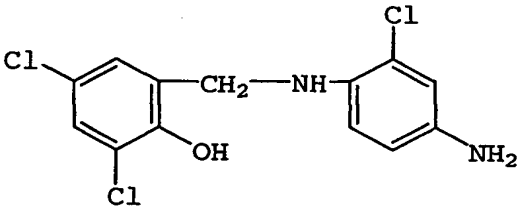


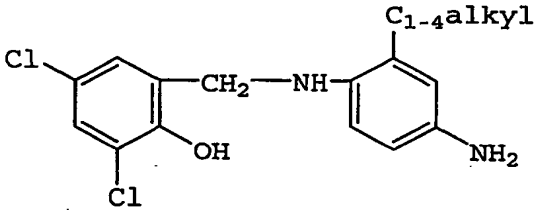


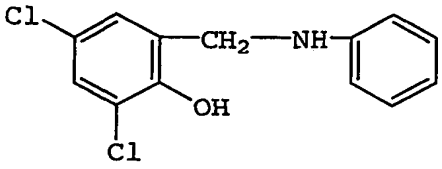


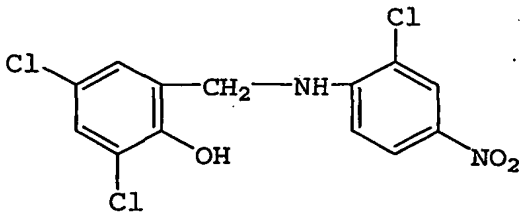












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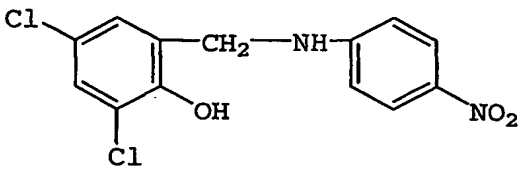

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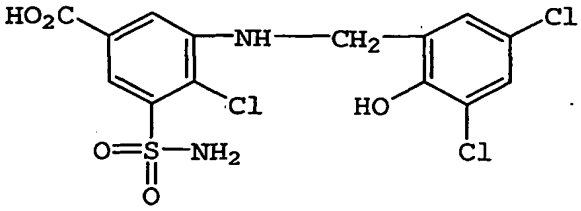

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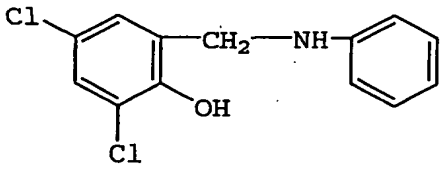

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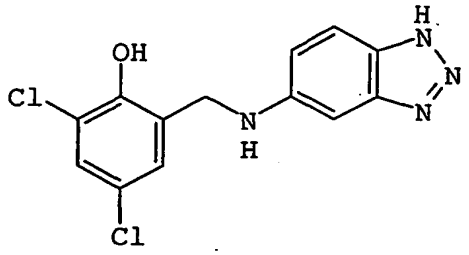
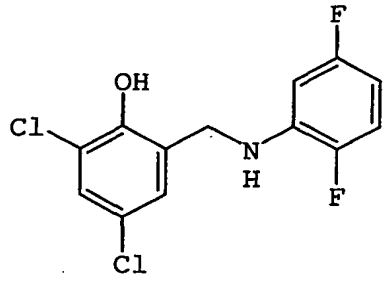
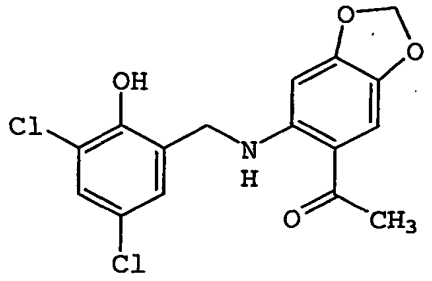
23. The method of claim 22 wherein the compound is selected from the group consisting of





, and



24. The method of claim 15 wherein the natural product extract is from *Actinomyces*.

25. The method of claim 15 wherein the peptide has a sequence selected from the group consisting of

Val-Glu-Thr-Asp-Val,
Glu-Thr-Asp-Val,
and mixtures thereof.

26. The method of claim 15 wherein the disrupting agent comprises Tat-nNOS (1-299).

27. The method of claim 1 further comprising administration of a therapeutically effective amount of an opiate analgesic to the mammal.

28. The method of claim 27 wherein the disrupting agent and opiate analgesic are administered simultaneously.

29. The method of claim 27 wherein the disrupting agent and opiate analgesic are administered sequentially.

30. The method of claim 27 wherein the amount of the opiate analgesic administered to the mammal is less than an amount of an opiate analgesic administered alone to achieve a predetermined reduction in pain.

31. The method of claim 27 wherein the opiate analgesic is selected from the group consisting of morphine, morphine sulfate, codeine, codeine phosphate, codeine sulfate, diacetylmorphine, morphine hydrochloride, morphine tartrate, diacetylmorphine hydrochloride, dextromethorphan hydrobromide, hydrocodone bitartrate, hydromorphone, hydromorphone hydrochloride, levorphanol tartrate, oxymorphone hydrochloride, oxycodone hydrochloride, fentanyl, meperidine, methodone, remifentanil, sulfentanil, fentanyl, alfentanil, and propoxyphene.

32. The method of claim 27 wherein the opiate analgesic comprises morphine.

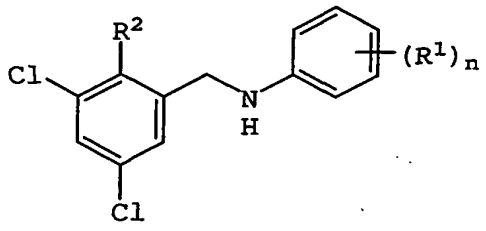
33. The method of claim 1 wherein the mammal is a human.

34. A composition comprising a disrupting agent of claim 15 and a pharmaceutically acceptable carrier.

35. The composition of claim 34 further comprising an opiate analgesic.

36. A method of treating a mammal suffering from a disease or condition wherein disruption of an interaction between Post Synaptic Density Protein 95 (PSD95) and neuronal nitric oxide synthase (nNOS) provides a benefit, said method comprising a step of administering to a mammal in need thereof a therapeutically effective amount of a PSD95-nNOS disrupting agent selected from the group consisting of

(a) a compound having a general structural formula



wherein R^1, independently, is selected from the group consisting of C_{1-4}alkyl, halo, CF_3, OCF_3, $C(=O)R^a$, $C(=O)OR^a$, $N(R^a)_2$, $C(=O)N(R^a)_2$, $NR^aC(=O)N(R^a)_2$, OR^a, SR^a, NO_2, CN, $SO_2N(R^a)_2$, SOR^a, SO_2R^a, and OSO_2CF_3,

R^2 is hydro or OH,

R^a, independently, is selected from the group consisting of hydro, C_{1-4}alkyl, aryl, and heteroaryl; and

n is an integer 0 through 4,

wherein two R^1 groups can be taken together with the carbon atoms to which they are attached to form an optionally substituted 5- to 7-membered aliphatic or aromatic ring, and optionally containing

one to three heteroatoms selected from the group consisting of oxygen, nitrogen, and sulfur,

or a pharmaceutically acceptable salt, solvate, or prodrug thereof;

(b) a natural product extract from a fungal or microbial source;

(c) a peptide having a sequence

A-B-C-D-E,

wherein A is null, Pro, or Val, having a terminal NH_2 group optionally acetylated or linked to Tat;

B is Glu, Gln, or Arg;

C is Thr;

D is Asp, Asn, or His;

E is Val, Leu, or Ile having a terminal $-CO_2H$ group,

and wherein B can be Asp, if D is Glu;

(d) Tat-nNOS (1-299); and

(e) mixtures thereof.

37. The method of claim 36 wherein the disease or condition comprises ischemic brain damage, a neurological disease, a neurodegenerative disease, or a psychiatric disorder.

38. The method of claim 37 wherein the disease or condition is selected from the group consisting of Parkinson's disease, epilepsy, seizures, stroke, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis, traumatic brain injury, muscular dystrophy, Duchenne muscular dystrophy, Becker muscular dystrophy, limb-girdle muscular dystrophy, and fascioscapulohumoral muscular dystrophy.

39. The method of claim 36 further comprising administering a therapeutically effective amount of a second therapeutic drug useful in the treatment of the disease or condition.

40. A method of reducing or reversing opiate tolerance comprising administering a therapeutically effective amount of an agent that disrupts an interaction between neuronal nitric oxide synthase (nNOS) and Post Synaptic Density Protein 95 (PSD95) to a mammal undergoing an opiate analgesic treatment.

41. A method of reducing or reversing opiate dependence comprising administering a therapeutically effective amount of an agent that disrupts an interaction between neuronal nitric oxide synthase (nNOS) and Post Synaptic Density Protein 95 (PSD95) to a mammal undergoing an opiate analgesic treatment.

42. A method of ameliorating pain associated with hyperalgesia comprising a step of administering to a mammal in need thereof a therapeutically effective amount of an agent that disrupts an interaction between neuronal nitric oxide synthase (nNOS) and Post Synaptic Density Protein 95 (PSD95).

43. A method of ameliorating pain associated with allodynia comprising a step of administering to a mammal in need thereof a therapeutically effective amount of an agent that disrupts an interaction between neuronal nitric oxide synthase (nNOS) and Post Synaptic Density Protein 95 (PSD95).

44. A method of alleviating pain in a mammal in need thereof, comprising administering to said mammal:

(a) an opiate analgesic compound in an amount insufficient to achieve optimal pain reduction, and

(b) a compound that disrupts an interaction between Post Synaptic Density Protein 95 (PSD95) and neuronal nitric oxide synthase (nNOS) in an amount sufficient to achieve optimal pain reduction in said mammal,

wherein pain is alleviated in said mammal.

45. In a method of alleviating pain in a mammal by use of an opiate analgesic compound, the improvement consisting of administering an effective amount of a compound that disrupts an interaction between Post Synaptic Density Protein 95 (PSD95) and neuronal nitric oxide synthase (nNOS), whereby an analgesic effect of the opiate analgesic compound is potentiated or the effective amount of the opiate analgesic compound is reduced.

46. In a method of controlling tolerance to an opiate analgesic compound, the improvement consisting of administering an effective amount of a compound that disrupts an interaction between Post Synaptic Density Protein 95 (PSD95) and neuronal nitric oxide synthase (nNOS), whereby an analgesic effect of the opiate analgesic compound is potentiated or the effective amount of the opiate analgesic compound is reduced.

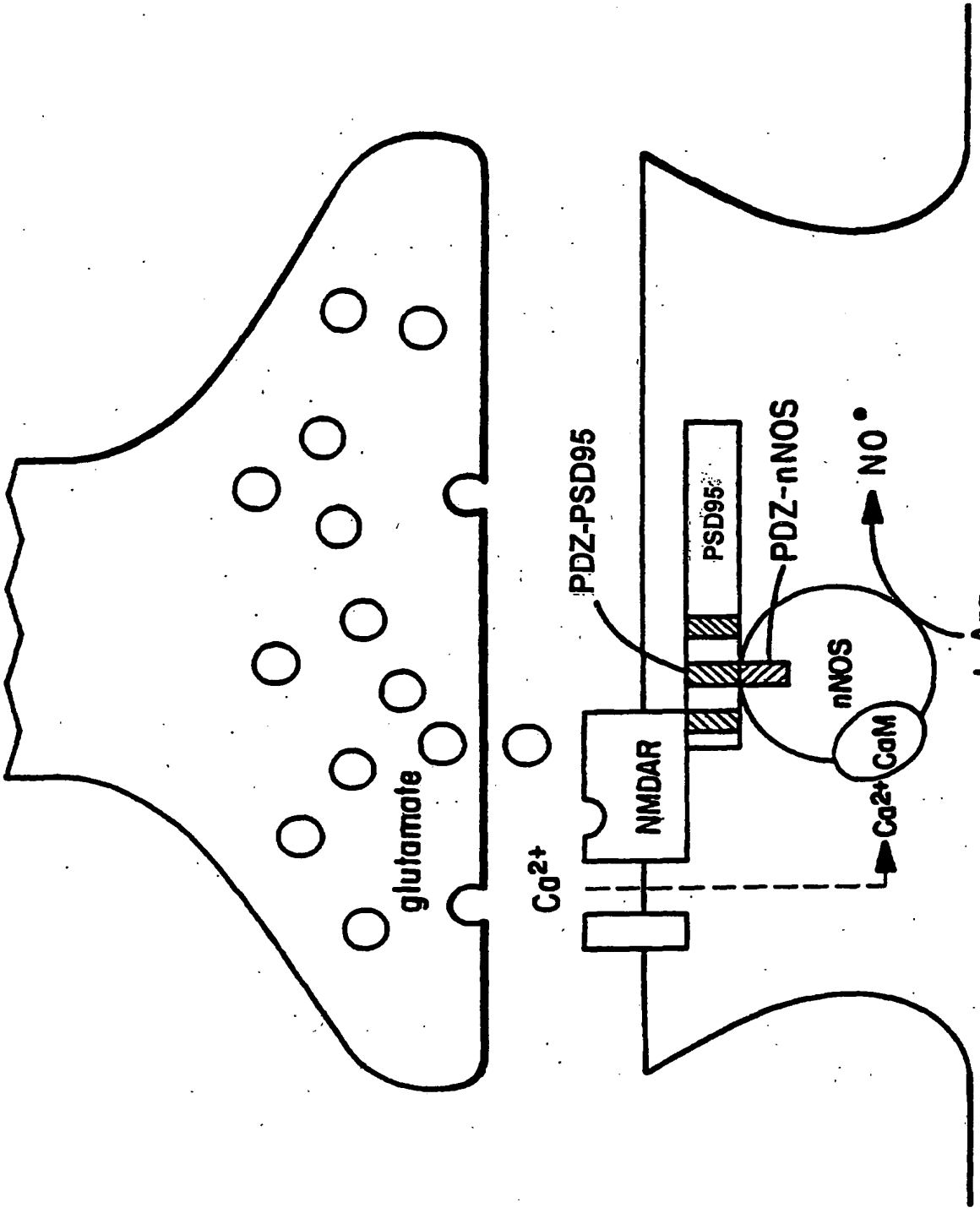

glutamate
Ca2+
NMDAR
PDZ-PSD95
PSD95
PDZ-nNOS
nNOS
CaM
Ca2+
L-Arg
NO•

Fig. 1

Fig 2

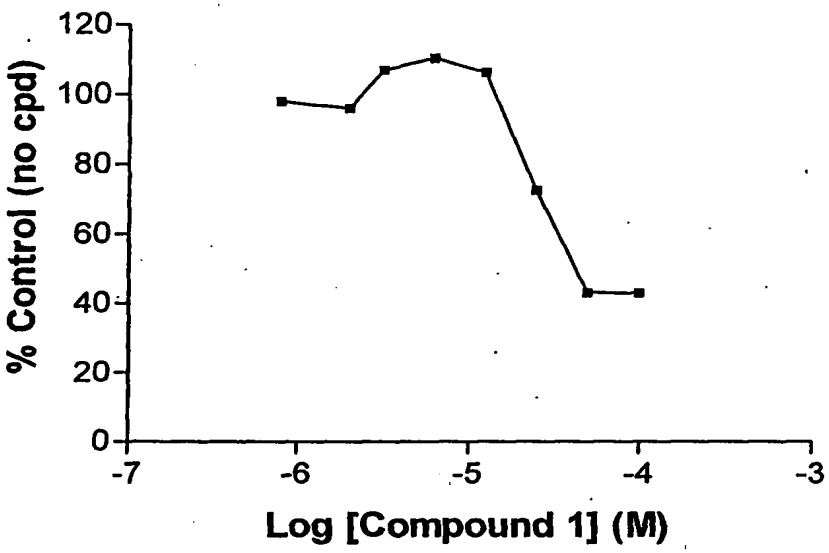


Fig 3

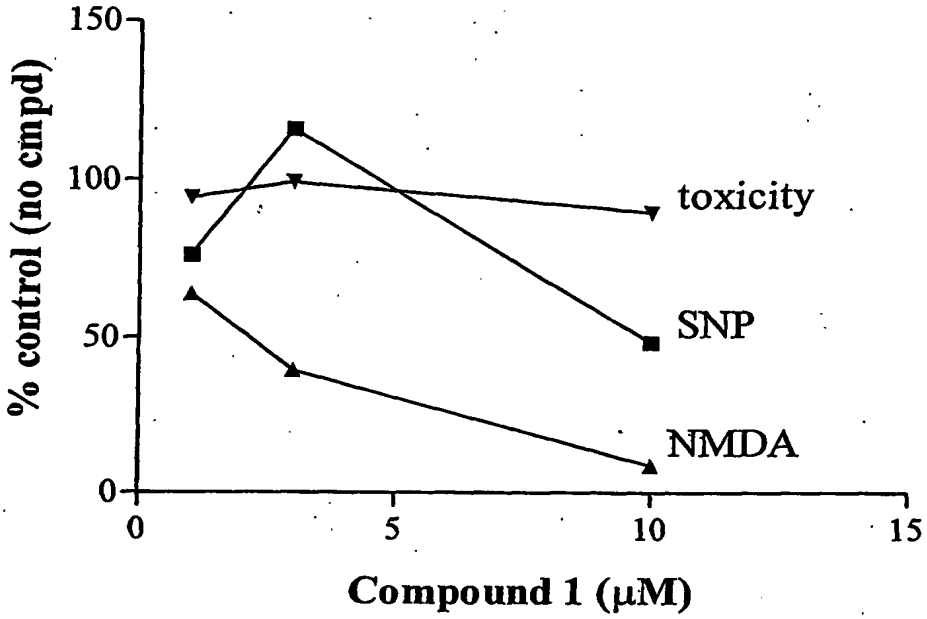

150
100
50
0
% control (no cmpd)
toxicity
SNP
NMDA
0
5
10
15
Compound 1 (μM)

Fig. 4

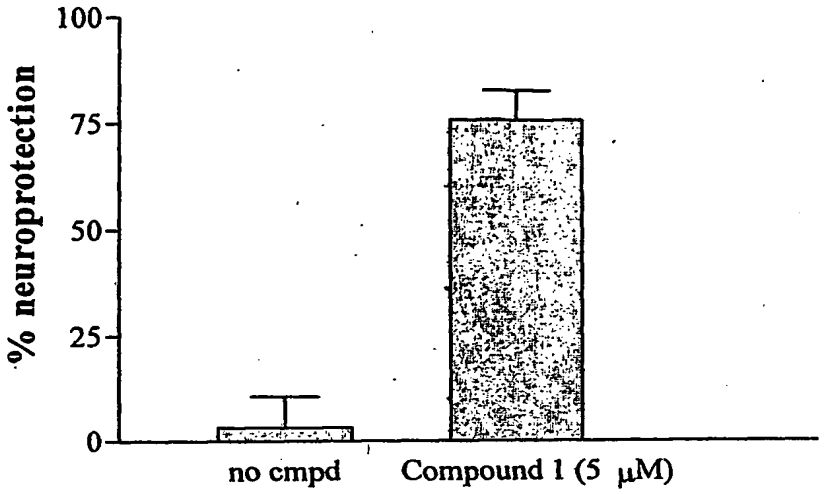


Fig. 5

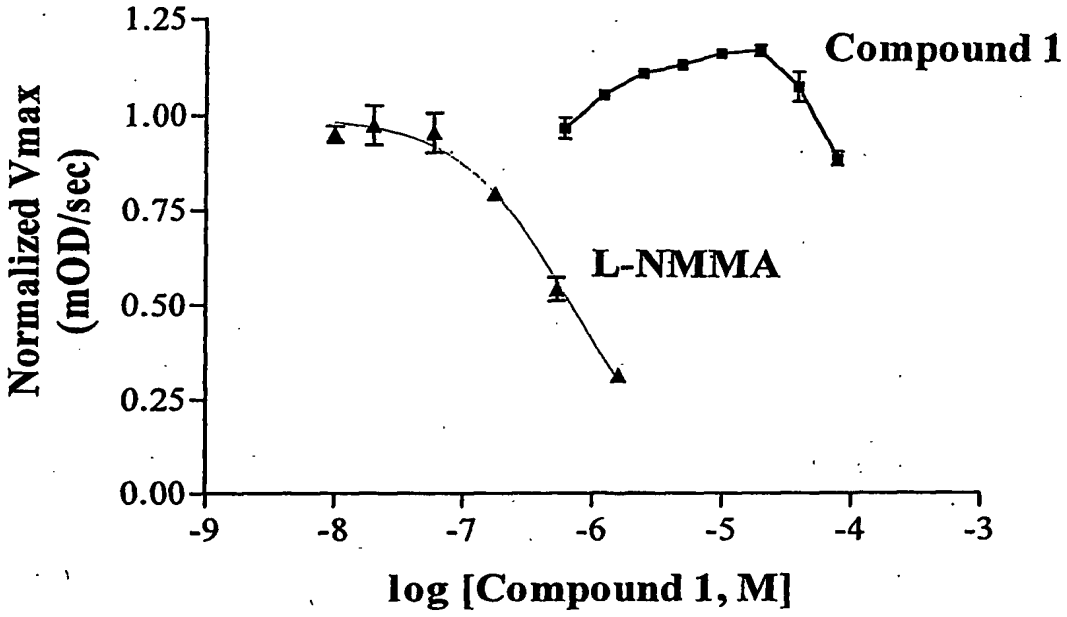

Normalized Vmax
(mOD/sec)
1.25
1.00
0.75
0.50
0.25
0.00
-9
-8
-7
-6
-5
-4
-3
log [Compound 1, M]
Compound 1
L-NMMA

Fig. 6

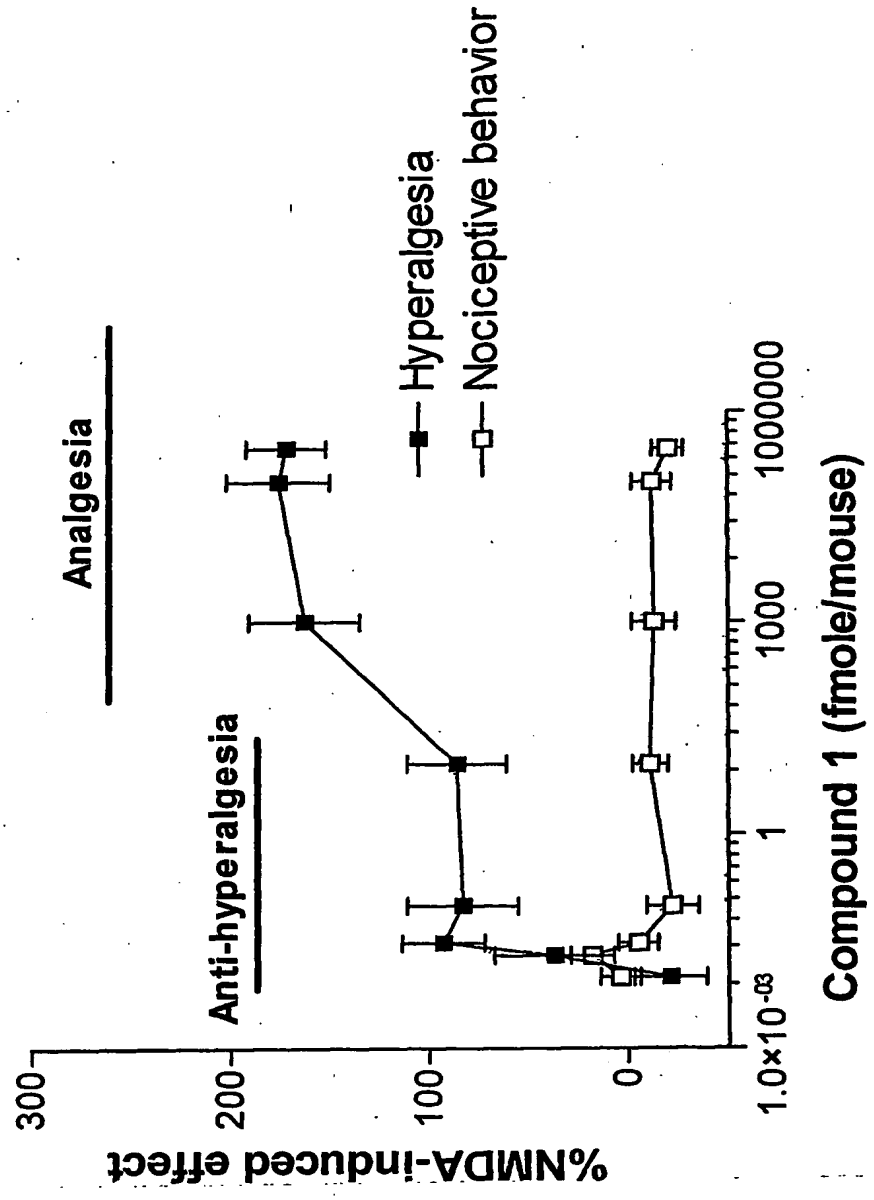

%NMDA-induced effect
300
200
100
0
Analgesia
Anti-hyperalgesia
Hyperalgesia
Nociceptive behavior
1.0×10-03
1
1000
1000000
Compound 1 (fmole/mouse)

Fig 7

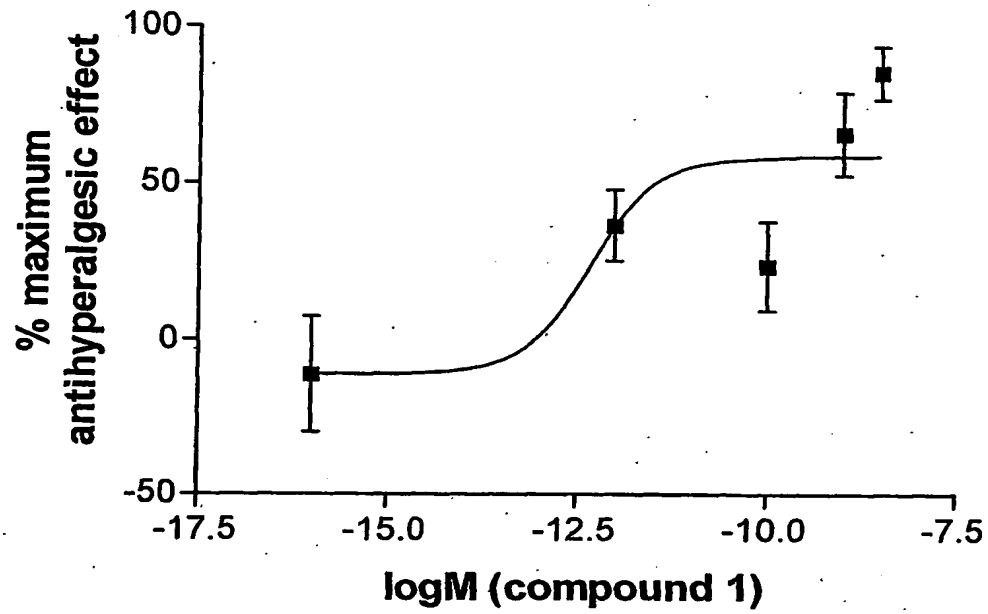

100
50
0
-50
% maximum antihyperalgesic effect
-17.5
-15.0
-12.5
-10.0
-7.5
logM (compound 1)

Fig 8

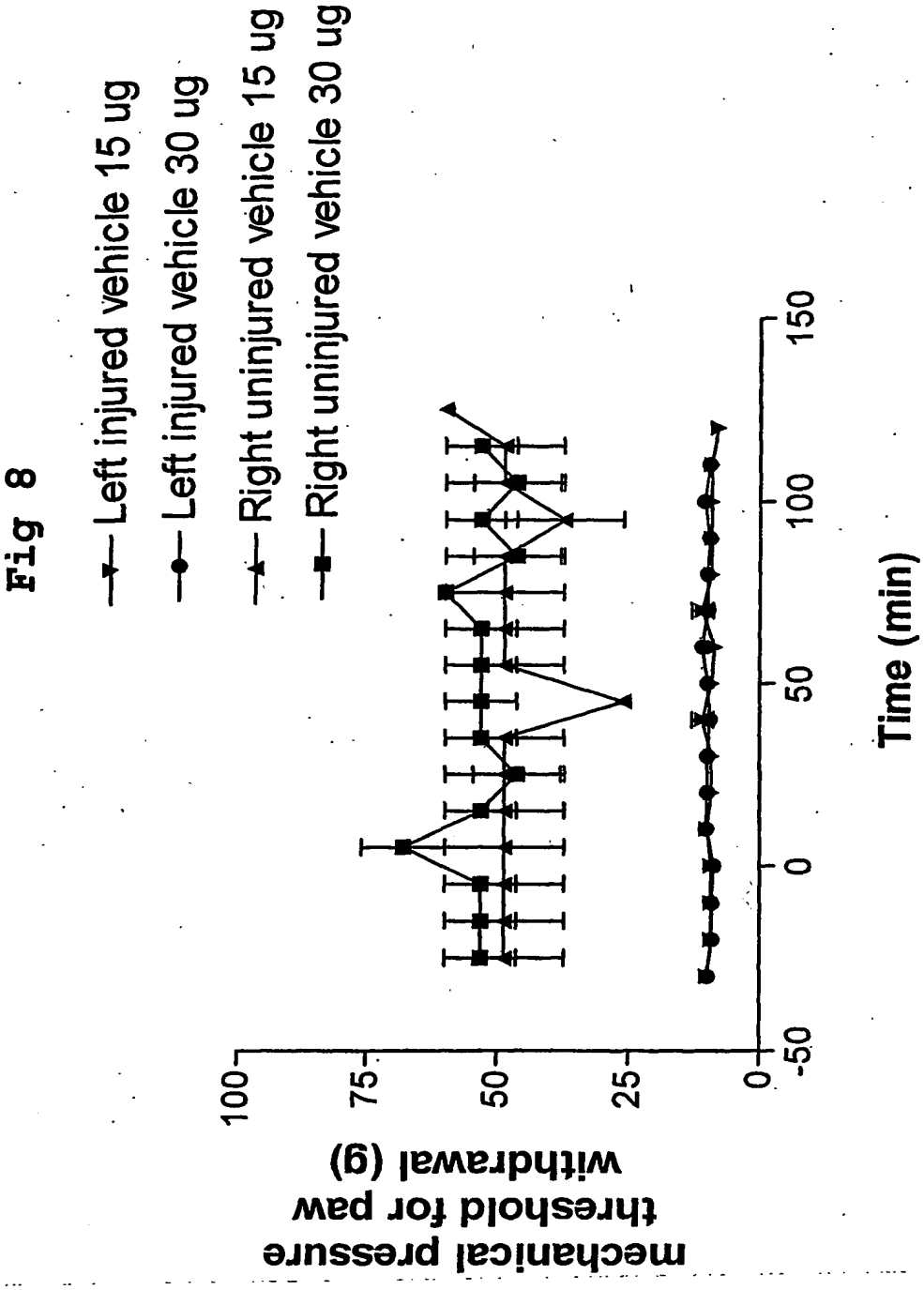

Left injured vehicle 15 ug
Left injured vehicle 30 ug
Right uninjured vehicle 15 ug
Right uninjured vehicle 30 ug
mechanical pressure threshold for paw withdrawal (g)
100
75
50
25
0
-50
0
50
100
150
Time (min)

Fig. 9

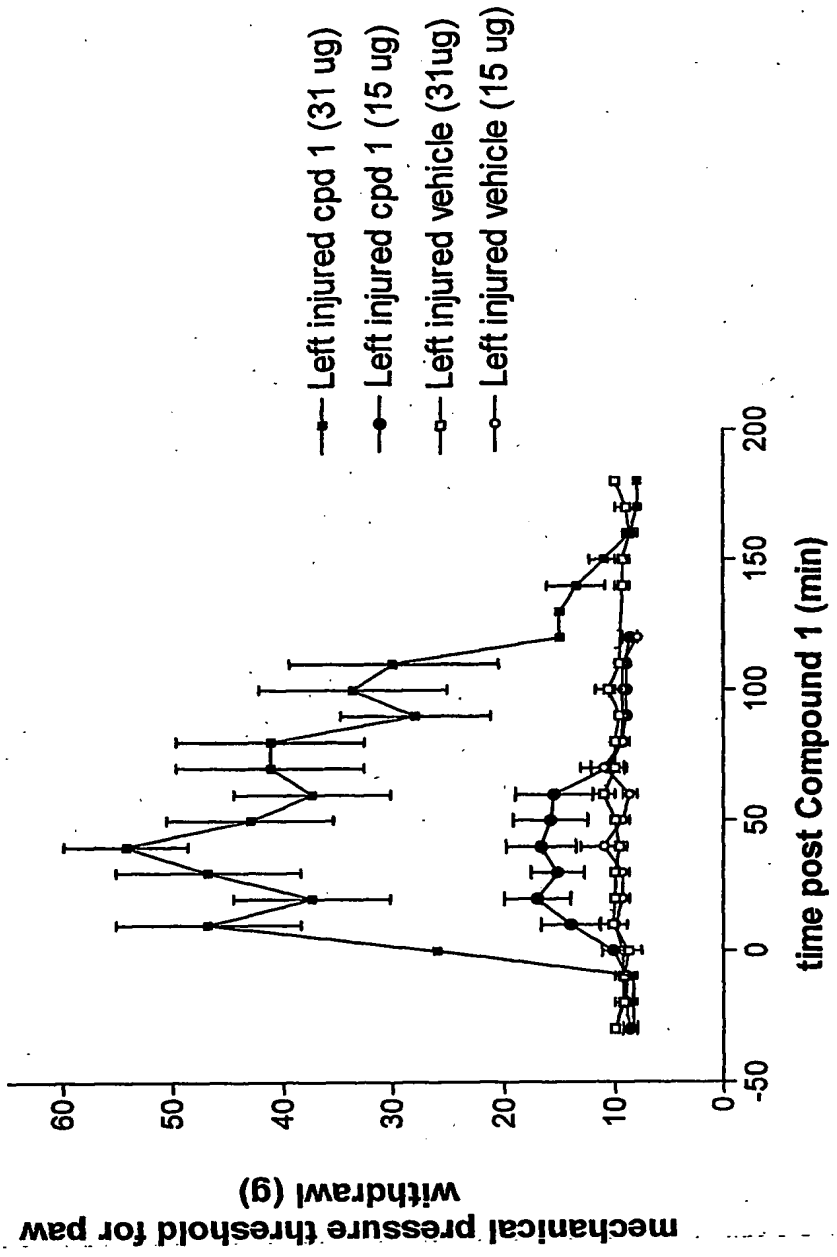

Left injured cpd 1 (31 ug)
Left injured cpd 1 (15 ug)
Left injured vehicle (31ug)
Left injured vehicle (15 ug)
mechanical pressure threshold for paw withdrawl (g)
time post Compound 1 (min)
0
10
20
30
40
50
60
-50
0
50
100
150
200

Fig 10

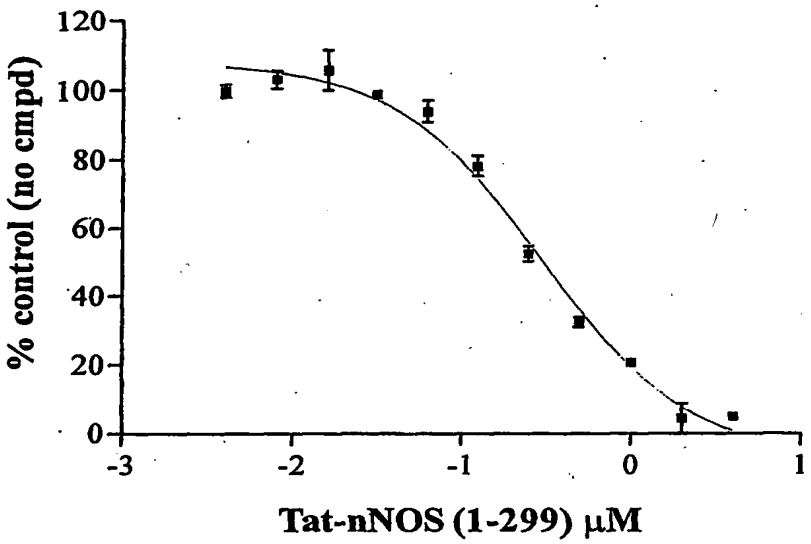


Fig 11

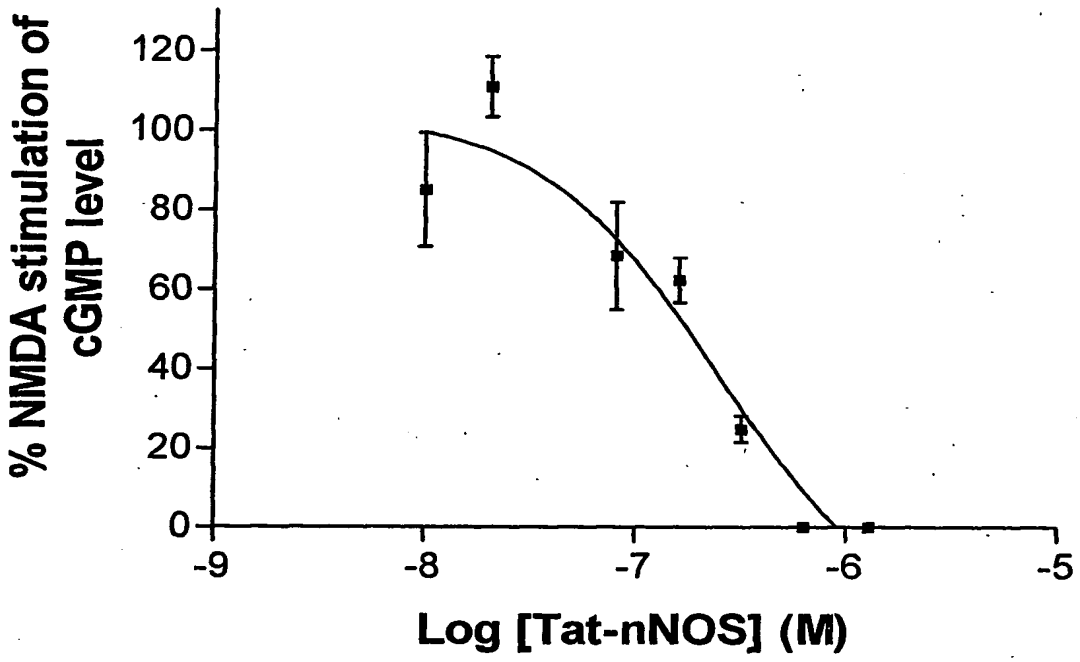

% NMDA stimulation of cGMP level
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
Log [Tat-nNOS] (M)

Fig 12

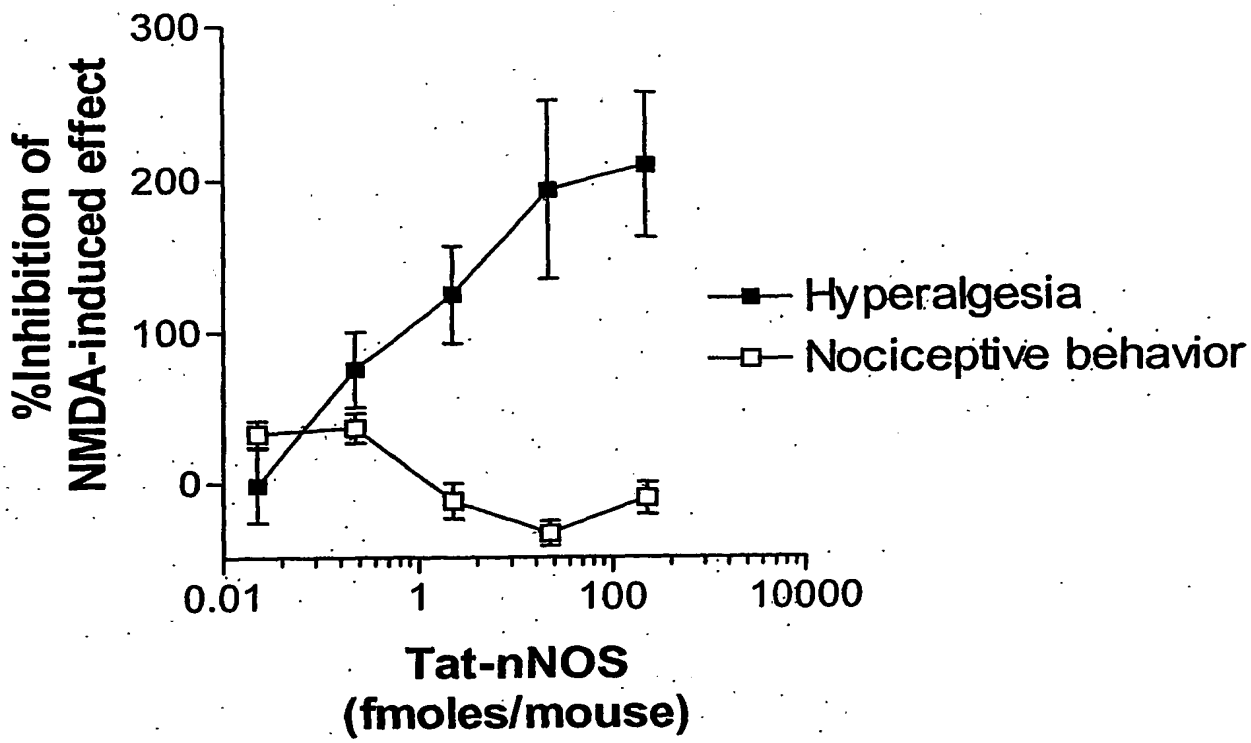
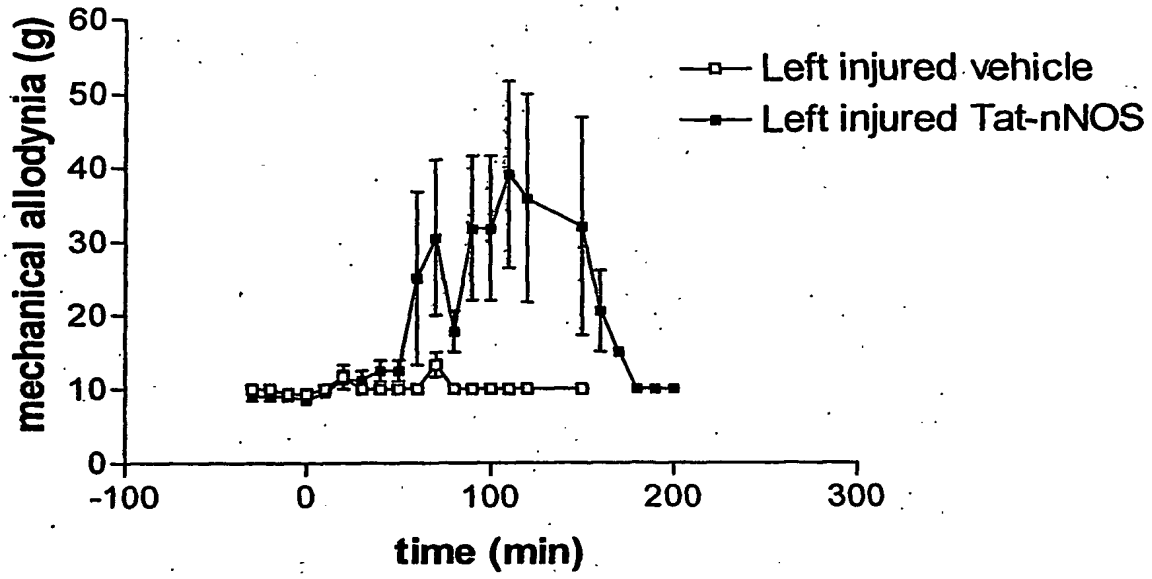

300
200
100
0
%Inhibition of NMDA-induced effect
0.01
1
100
10000
Tat-nNOS (fmoles/mouse)
Hyperalgesia
Nociceptive behavior

Fig 13


mechanical allodynia (g)
60
50
40
30
20
10
0
-100
0
100
200
300
time (min)
Left injured vehicle
Left injured Tat-nNOS

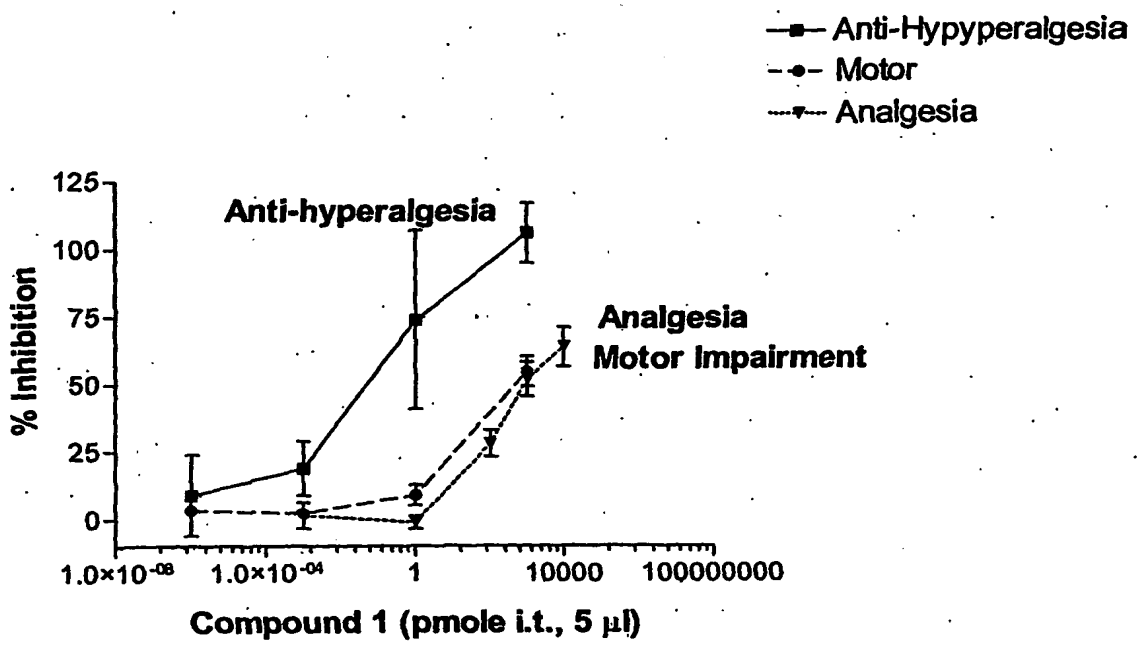

Anti-Hypyperalgesia
Motor
Analgesia
Anti-hyperalgesia
Analgesia
Motor Impairment
% Inhibition
125
100
75
50
25
0
1.0×10-08
1.0×10-04
1
10000
100000000
Compound 1 (pmole i.t., 5 µl)

Fig. 14

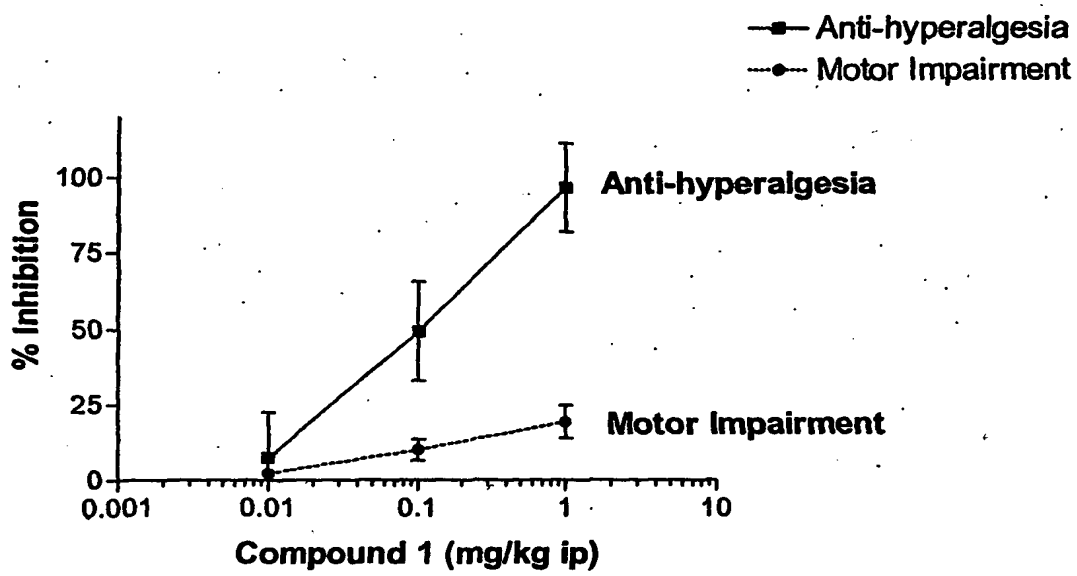

Anti-hyperalgesia
Motor Impairment
% Inhibition
100
75
50
25
0
0.001
0.01
0.1
1
10
Compound 1 (mg/kg ip)

Fig. 15

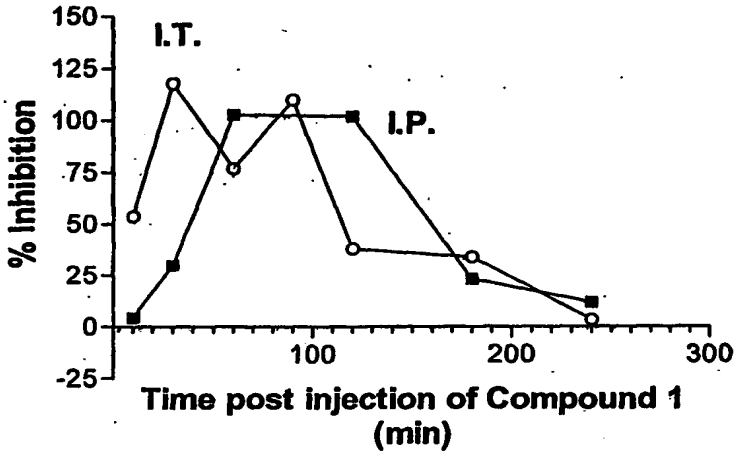

150
125
100
75
50
25
0
-25
% Inhibition
I.T.
I.P.
100
200
300
Time post injection of Compound 1
(min)

Fig. 16

SEQUENCE LISTING

<110> Janosky, et al

<120> AGENTS THAT DISRUPT PSD95-nNOS INTERACTION, COMPOSITIONS CONTAINING THE SAME, AND THERAPEUTIC USES THEREOF

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<170> PatentIn version 3.3

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